	Inventaris Wob-verzoek W16-01								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 2015104								
1	Aanvraagformulier				x		x	x	
2	Niet-technische samenvatting	x							
3	Projectvoorstel			x					
4	Bijlage beschrijving dierproeven 1			x					
5	Bijlage beschrijving dierproeven 2			x					
6	Bijlage beschrijving dierproeven 3			x					
7	Bijlage beschrijving dierproeven 4			x					
8	Bijlage beschrijving dierproeven 5			x					
9	Ontvangstbevestiging				x		x	x	
10	Vervolgbrief aanvraag				x		x	x	
11	Verzoek DEC-advies				x		x	x	
12	DEC-advies				x		x	x	
13	Brief antwoorden				x		x	x	
14	Advies CCD		x						x
15	Beschikking en vergunning				x		x	x	
16	Mail ontvangstbevestiging 22-5-2015				x		x	x	
17	Mail DEC aanpassing 5-6-2015				x		x	x	
18	Mail aanpassing tekst 11-6-2015				x		x	x	
19	Mail DEC aanpassing 12-6-2015				x		x	x	
20	Mail dubbele betaling 18-6-2015				x		x	x	

1

26 MEI 2015

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☑ Ja > *Vul uw deelnemernummer in* 80101 Nederlands Herseninstituut-KNAW
☐ Nee > *U kunt geen aanvraag doen*

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	KNAW
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	5 4 6 6 7 0 8 9

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	
Postbus	Postbus 19121
Postcode en plaats	1000GC Amsterdam
IBAN	NL33DEUT0546900054
Tenaamstelling van het rekeningnummer	Nederlands Herseninstituut

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	Group Leader
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.6 *(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.

(Titel) Naam en voorletters	☑ Dhr. ☐ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	[redacted]

1.7 Is er voor deze projectaanvraag een gemachtigde?

☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*

☑ Nee

2 Over uw aanvraag

2.1 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 3

☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.2

☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Vul uw vergunde projectnummer in en ga verder met vraag 2.3

2.2 Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?

☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier

☑ Nee > Ga verder met vraag 3

2.3 Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?

☑ Nee > Ga verder met vraag 3

☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?	Startdatum: 01-07-2015 Einddatum: 01-07-2020
3.2 Wat is de titel van het project?	Developing strategies to promote repair or plasticity of the central and peripheral
3.3 Wat is de titel van de niet-technische samenvatting?	Ontwikkeling van strategieën om de regeneratie van zenuwweefsel te bevorderen
3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC: DEC-KNAW Postadres: [redacted] Amsterdam E-mailadres: [redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- ☑ Nieuwe aanvraag Projectvergunning € 741,00 Lege
- ☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

- ☑ Via een eenmalige incasso
- ☐ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☐ Melding Machtiging
- ☒ Appendixen 5 maal

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted]
Plaats	Amsterdam
Datum	18-05-2015
Handtekening	[redacted]

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	NVWA80101
1.2	Provide the name of the licenced establishment.	Netherlands Institute for Neuroscience
1.3	Provide the title of the project.	Developing strategies to promote repair or plasticity of the central and peripheral nervous system

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

X Basic research

☐ Translational or applied research

☐ Research into environmental protection in the interest of human or animal health or welfare

☐ Research aimed at preserving the species subjected to procedures

☐ Higher education or training

☐ Forensic enquiries

☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Dysfunction of the brain or spinal cord (collectively referred to as the central nervous system [CNS]) or of the peripheral nervous system (PNS) due to neural degeneration or traumatic injury affects millions of people worldwide and has a significant physical, emotional, and socio-economical impact. There are many different causes for neural degeneration or injury ranging from genetic causes, toxic insults, traffic and sports-related incidents, community violence and work-related incidents. Nervous system degeneration primarily entails the death of specific populations of neurons themselves (e.g. the loss of dopaminergic neurons in patients with Parkinson's disease) and/or the interruption of nerve projections and the associated loss of synaptic contacts (e.g. in patients with brain, spinal cord or peripheral nerve injury). When nerve cells are lost, these cells are not replaced by new cells (as opposed to cells in many other organs). In the CNS damaged, interrupted nerve tracts normally do not regrow, while in the PNS injured nerve tracts do regrow but only to some extend and after severe lesions most nerve fibers do not re-innervate the target. The failure to regenerate is due to the induction of the expression of growth inhibitory factors, for instance at the site of the injury (in the neural scar formed after a CNS lesion), and the lack of the production of pro-regenerative molecules (e.g. neurotrophic factors or transcription factors). In the injured CNS the balance shifts to growth inhibition and, therefore, regeneration is very poor in the CNS. However, some recovery of function may occur due to the induction of growth of intact neighboring axon pathways. This process is usually referred to as plasticity and is also an important mechanism for partial recovery of function and repair. In the PNS the situation is slightly better than in the CNS, e.g. Schwann cells in an injured peripheral nerve do support nerve fiber growth, but after longer times of denervation these cells deteriorate and do not support growth anymore.

Currently there are no effective treatments for patients that have sustained injury to the CNS. For instance, Parkinson patients are treated pharmacologically with L-DOPA to supplement the loss of dopamine. This treatment temporally alleviates some of the symptoms of the disease but it is not a regenerative treatment. Patients with a lesion of the PNS can be treated by a neurosurgeon, however, regeneration and functional recovery of a nerve following surgical intervention is almost never complete. Therefore, what is urgently needed is more fundamental knowledge on (i) which molecules and genes determine the loss of neurons, (ii) which factors control in the outgrowth of neuronal projections, and (iii) which factors limit the regeneration of injured neurons. This knowledge will be tested for their potential to be used as genuine regenerative treatments that (1) promote neuronal survival in order to prevent neurons from degeneration, (2) promote nerve fiber outgrowth and the formation of new synaptic connections of lesioned or spared nerve tracts by adding growth promoting factors or the removal of inhibitory factors. We focus our research both on genuine regeneration of injured nerve tract and on structural remodeling of spared nerve tracts (also referred to in the field as structural plasticity) because structural remodeling of the intact fibers may also have a significant positive impact on functional recovery. For many of our project we have used human nervous tissue as the starting material of our screens and as tissue to start to refine our gene transfer methods.

There is general agreement in the research field of neurodegeneration and regeneration that the current neurosurgical intervention strategies have reached optimal refinement. Therefore these interventions will not lead to the level of repair that is required for patients with nervous system injury to allow a return to a normal life. Hence the long-term aim of the research in this field should be on developing effective regenerative treatments. For this it is essential to identify factors (genes, molecules, cell types) that are pivotal in the survival of neurons, the regeneration of injured nerve projections and the formation of new synapses. We and others have already identified two cell types *[Schwann cells (SCs) and Olfactory ensheathing glia cells (OEGs); reviewed in Roet and Verhaagen, Experimental Neurology 2014]* and several molecules that either promote neuronal survival, axon regeneration or plasticity *[growth factors, wnts, transcription factors; reviewed in Fagoe et. al. 2014]* or hamper axon regeneration or plasticity *[extracellular matrix molecules, e.g. semaphorins; reviewed in Mecollari et. al. Frontier in Neuroscience 2014].* Some of these cell types and factors have subsequentially been tested for their neuroprotective or growth-promoting effects in lesion models in experimental animals. Specific examples of work from our own laboratory include the profound pro-regenerative effect of transplanted, genetically modified, OEGs in a spinal cord lesion *(Ruitenberg et.al. Journal of Neuroscience 2003)*, of the growth factor BDNF on the survival of rubrospinal neurons *(Ruitenberg et al Neurobiology of Disease 2004)* and of the growth factor GDNF on motor axon outgrowth *(Eggers et. al. Molecular Cellular Neuroscience 2008, Hoyng et. al. Experimental Neurology 2014a)*. Recent, as yet unpublished, discoveries from our group include the observation that the signaling molecule Wnt5a promotes nerve fiber outgrowth *(van Vliet, unpublished)* and that functional neutralisation of Semaphorin3A (a molecule obstructing regeneration) promotes repair of the injured spinal cord *(Mecollari, unpublished)* and enhances plasticity in the brain *(Vo et. al. Molecular and Cellular Neuroscience 2013, and unpublished observations in collaboration with Daniela Carulli and Tommasso Pizzurosso, Italy)*. Moreover we have identified molecules that induce degeneration of neurons, specifically the dopaminergic neurons that die in Parkinson's disease (*Bossers et al Brain Pathology 2009; Korecka and Moloney et al unpublished*).
As a means to express pro-regenerative molecules in damaged neural tissue in order to study their functional involvement, we have developed advanced and innovative gene transfer strategies (based on adeno-associated and lentiviral vectors, some of which have clinical potential; *reviewed in Mason et.al. Current Gene Therapy 2011)*. Gene transfer with viral vectors is chosen as a primary approach because it is a very powerful method to locally express a gene and study its function in the injured nervous system. Moreover eventually this strategy may be applicatble clinically since in the last 10 years an the adeno-associated viral (AAV) vector has gained increasing acceptance as a clinical gene therapy platform. Viral vector-mediated gene transfer was optimized for gene transfer in injured neurons by testing which serotype *(Mason et.al. Molecular Therapy 2010, Blits et.al. J. Neuroscience Methods 2011; Korecka et. al. Viral vectors 2011)* and route of delivery *(Fagoe et.al. Neuromethods 2015)* was the best. In addition, we also combined tissue transplantation with gene transfer e.g. in a study where we used genetically modified nerve autografts to repair injured peripheral nerves *(Hoyng et.al. Experimental Neurology 2014)*. Although gene therapy is in our view a very powerful way to study gene function, it may also be a clinically applicable strategy to promote repair the use of small molecules (inhibitors, agonist; that is pharmacological intervention). This is certainly an option that we want to pursue if this would be more rational.

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The main aim of this project is to unravel the fundamental cell and molecular biological mechanisms that underlie the failure of the nervous system to repair itself. Specifically the project aims to identify molecules that promote or inhibit the neuroregeneration process, to overexpress or neutralize these molecules and to study the effects of these interventions on the recovery process.
The laboratory and scientific infrastructure needed is available at the Netherlands Institute for Neuroscience which makes this research highly feasible. In addition to the scientific achievements of the group summarized above, the group has a long-term internationally recognized track record in

neuroregeneration research. This is illustrated by the fact that the group published over 190 papers on this topic, received national and international funding and was positively evaluated by the KNAW-audit committee in 2012. The group has been part of several European consortia and has international collaborations with groups in e.g. Cambridge, Turin, and Perth.
Taken together, and based on the available data, know-how, and infrastructure as summarized above, we expect that in the next five years it will be realistic to firmly establish the role of at least three and perhaps six new molecular targets in the neural repair process. We also collabrate with clinicians, e.g. at the department of neurosurgery at the LUMC, to allow translation of our research to the clinic.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

This project is important for two reasons: 1. It results in fundamental insights into the mechanisms that inhibit or promote regeneration, and 2. These results form the basis for repair strategies that will eventually be applied to promote repair in patients.

Apart from pharmacological treatment (e.g. with L-DOPA in Parkinson patients which results in temporary relief of symptoms) or neurosurgical repair of an injured peripheral nerve (which results mostly in only partial recovery of function) there are no effective treatments available for patients with nervous system degeneration/injury. Individuals with neural injury therefore suffer from a life-long disability and many are usually dependent on outside care and/or are bound to a wheelchair. There is an urgent need for treatments that promote nervous system repair and full functional recovery.

In the current project we develop strategies that have the aim to promote neuroprotection and/or neuroregeneration with the long-term goal to lead to medically applicable treatment options for patients with brain, spinal cord or peripheral nerve injuries. These regenerative treatments will be beneficial for individual patients and for society as a whole because effective treatments may allow patients to rehabilitate and improve their quality of life.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

To achieve the aims described above we make use of a four step strategy (our "target discovery pipeline") developed by us that includes (1) large gene expression and proteomics screens on control (non-damaged) or damaged human neural tissue or on control and damaged tissue of experimental animals to identify molecules involved in the neural repair process, (2) bioinformatical analysis of the resulting gene and protein data sets in combination with published datasets to identify the most promising targets, (3) to perform bioassays for neuronal survival, axon outgrowth and synapse formation to confirm a functional role of the identified candidate targets using primary neural cells, and finally (4) gene and cell therapy studies in (transgenic) animal models of neurodegeneration and injury to test the mechanism of action and/or effectiveness of a final set of most promising targets. For this, we have developed and tested advanced viral vector-mediated gene transfer technology that allows us to express genes in vivo in injured neurons or glia cells. We are also in the process of developing regulatable viral vectors that are based on a novel immune-inert transactivator that allows antibiotic-mediated control over transgene expression in vivo (Hoyng et al Experimental Neurology 2014b). Our approach has shown to result in valuable fundamental knowledge and form the foundation for novel, potential repair strategies in patients.

Thus, the overall strategy of this project consists of a number of distinct steps aiming at target discovery for neural protection and repair using molecular screens and bioinformatics from published data sets and from data obtained from our own models (procedure 3.4.4.1), target validation in vitro by means of

bioassays (procedure 3.4.4.2), and investigation of target efficacy in vivo in (transgenic) animal models (procedures 3.4.4.3; 3.4.4.4; 3.4.4.5) of neural degeneration and regeneration. We refer to this strategy as our "target-discovery strategy". Below we will describe how we execute each of these steps and what is required in terms of types of animal experiments.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Molecular screens. The first step in our target finding approach are molecular screens, using microarray or RNA-sequencing, which are performed on unaffected control neural cells and on injured neural cells. The comparison yields insights into the molecular differences between intact, non-injured neural cells and injured neural cells. This step is performed on several different sources of tissue, including human nervous system tissue obtained from the Netherlands Brain Bank, from the operation theatre (both do not require experimental animals), or from tissue obtained from experimental animals after a lesion of e.g. a spinal cord or a peripheral nerve lesion or from transgenic animals. Animal experiments are needed to obtain tissue from animals with or without a neural lesion (procedure 3.4.4.1; for neural lesions see "injury models" below).
Bioassays. Bioassays are essential for two reasons. First, the molecular screens usually result in many different interesting targets and only the most promising molecules need to be selected for testing in injury models. We have large-scale bioassays to measure the effect of overexpression or knockdown of target on e.g. neuronal survival and axon outgrowth, two processes directly relevant to neuroprotection and repair. Second, viral vectors used for gene transfer have to be tested for efficacy on primary neurons or glia cells before any application in vivo. For both applications we need to culture primary neurons or glia cells derived from rat or mouse embryo's or adult mice or rats (procedure 3.4.4.2).
Injury models. It is important to test our targets under different circumstances because certain parts of the nervous system regenerate to a certain extend (e.g. the peripheral nerve) while other parts (the spinal cord) do hardly regenerate. Therefore we need different types of injury. The efficacy of a selected target is determined either in an animal model for neural injury (in case we expect an effect on the regeneration process) or in a naïve animal (in case we hypothesize that a molecular target is involved in neurodegeneration or synapse loss as is e.g. the case for some chemorepulsive proteins). The lesion is produced e.g. by means of transection of a nerve tract in the spinal cord (e.g. the dorsal column or the corticospinal tract) or transection of a peripheral nerve. Type of animal experiment: lesioning of the brain, spinal cord or peripheral nerve is part of procedures 3.4.4.3, 3.4.4.4, 3.4.4.5.
Viral vector or cell-mediated gene delivery of a target. Targets identified in the molecular screens and functionally validated in bioassays are delivered to the nervous system by means of viral vectors (direct in vivo gene transfer) or by means of cell transplants that have been exposed to a viral vector in vitro ("ex vivo gene transfer"). The animal experiment required here is: Stereotactic injection of a viral vector or of cells in a specific brain nucleus, in the spinal cord, in a spinal ganglion, or in the peripheral nerve or the muscle. The chosen injection site is dependent on the specific target and on the goal of the experiment (procedures 3.4.4.3 - functional and histological analysis). Moreover we need to test the performance of most new produced batches of viral vectors in vivo on a small number of animals (procedure 3.4.4.4).
In the ideal situation, the efficacy of the viral vector or cell-mediated gene delivery of a target will be carried out in one of the injury models to test the effect of a selected target on neuronal degeneration or regeneration. However, for some experiments WT or transgenic mice may be used, for instance when a target is implicated in neuronal degeneration or neuroplasticity. To test the effects of the application of a target two main read-out parameters are used in parallel: functional and morphological tests (part of procedure 3.4.4.4).
Functional analysis. The efficacy of a treatment may evaluated by means of electrophysiological and/or functional approaches. With electrophysiology, the return of compound motor action potentials (CMAPs) and spinal evoked potentials are evaluated in time after the injury. Functional behavioral tests include the narrow beam test, gridwalk test, grip test, open field test, rope test, cylinder test, pole jump test, kinematic gait analysis test, catwalk gait analysis and foot flick test (part of procedure 3.4.4.3.).
Morphological analysis. For this, tissue for histological analysis is obtained following perfusion fixation of an experimental animal at different times after inflicting the injury. The efficacy of a particular treatment or target is analyzed at the level of cell survival, degree of axon outgrowth, scar formation and synapse formation. Type of animal experiment: Perfusion of rats and mice. (part of procedure 3.4.4.3.).

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection

points.

In this project we identify and characterize target molecules that promote or inhibit repair of the CNS or PNS using bioassays and in vivo models. Some targets have been identified based on previous discoveries by ourselves or of others, e.g. we work on a number of growth factors (NGF, BDNF, GDNF) and repulsive guidance molecules (Semaphorins) in the context of regeneration, structural plasticity and synapse formation. The "target discovery strategy" described above has already served to identify a number of new targets by ourselves. Typically, this pipeline consist of 4 steps that logically follow each other and progresses from "target identification" to "efficacy studies" in an animal model. For each target a go/no go decision is made whether or not the target should be studied at the next step. Primary target identification is based on human tissue obtained from the Netherlands Brain Bank or from the operation theatre obtained in collaboration with neurosurgeons. In some cases we perform genome wide gene expression studies on tissue from (transgenic) mice or rats using our injury models.

The logical structure of these different phases is best illustrated by an example from our recent research: Step 1/2: screening (step 1) and bioinformatics (step 2) - the protein Wnt5a was selected from a small group of highly upregulated molecules in a screen of injured human peripheral nerve tissue that was removed by the neurosurgeon during a nerve repair operation. Step 3: The effect of Wnt5a upregulation was studied in primary neuronal cell cultures and we found that Wnt5a significantly promotes neurite outgrowth. Therefore, Wnt5a was taken to step 4: testing of the effect of Wnt5a overexpression in vivo in a rat peripheral nerve injury model using both functional and histological read-outs. Moreover, in a Wnt5a knock-out mice introduced in the Netherlands Institute for Neuroscience from a group in Japan (Hiroaki Honda, Hiroshima University) we are currently studying in vivo effects of Wnt5a on axon regeneration. Taken together, for each target go/no go decisions are made based on the performance in each specific stage of the project as illustrated above for Wnt5a.

It is important to note, that there is some overlap between the animal studies described in this project and those in earlier DEC-approved protocols. After a license for this project has been obtained, all experiments will formally be executed under this new license.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Molecular screens: Sacrifice of mice or rats following a lesion and/or intervention with a viral vector to obtain neural tissue for molecular screens
2	Ex vivo bioassays: Sacrifice of embryos of mice or rats or of adult mice or rats to obtain tissues for cell culture and bioassays
3	Injury models (functional and histological analysis): Injection of cells or viral vectors in lesioned animals
4	Testing of the quality of viral vector batches
5	Monitoring and testing of novel genetically modified mice
6	
7	
8	
9	
10	

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	NVWA 80101
1.2	Provide the name of the licenced establishment.	Netherlands Institute for Neuroscience
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: # 1 Type of animal procedure: Molecular screens: Sacrifice of mice or rats following a lesion and/or intervention with a viral vector to obtain neural tissue for molecular screens

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

A primary aim of our research is to unravel new mechanisms that govern or hamper neuroregeneration or neuroplasticity. To achieve this we perform, in addition to analysis of patient material, molecular screens on neural tissue after a lesion to identify or mechanistically validate molecules that are potentially involved in the regeneration process or in the processes that hamper regeneration.

The brain, spinal cord or peripheral nerve lesions will be performed by means of well-established neurosurgical procedures. The types of lesion are chosen because they represent clinically relevant injuries. In different parts of the CNS different processes underlie degeneration and regeneration and it is therefore important to study different parts of the CNS (brain, spinal cord, spinal ganglion, peripheral nerve) and innervated target muscles. In some instances we will perform a lesion and inject a viral vector encoding for a gene of interest ("a target") or a molecule of interest (e.g. a transcription factor, Wnt5a or Semaphorin3A). At a particular post-lesion interval animals will be killed and tissue will be dissected out for a molecular screen, e.g. a microarray screen, RNA-sequencing or proteomics analysis. This specific aim is to provide quantitative insight in the changes in mRNA or protein levels that occur in neural tissue after a lesion and/or after applying a specific treatment (e.g. viral vector-mediated gene transfer of a specific target gene).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

This procedure can consist of the following steps:

1) *Application of a lesion to the brain, spinal cord or peripheral nerve*. Under adequate anaesthesia and perioperative analgesia, the brain, spinal cord, or peripheral nerve is exposed and a unilateral or bilateral lesion is made by means of damaging a nerve tract.
    a) *Brain*: injection of a neurotoxin systemically (I.P.) or intracranially, or by means of inflicting mechanical injury with micro-scissors *(max 1x).*
    b) *Spinal cord*: At cervical or thoracic or lumbar level, access to the spinal cord is gained and a uni- or bilateral lesion of the rubrospinal tract, corticospinal tract, dorsal column or dorsolateral columns is performed by means of microscissors *(max 1x).*
    c) *Peripheral nerve*: After gaining access to the peripheral nerve lesion site, unilateral crush or transection or spinal root avulsion is performed *(max 1x).*
2) *(Optional) Viral vector or cell-mediated gene delivery of a target or injection of the target molecule itself.* Under adequate anaesthesia and postoperative analgesia a (stereotaxic) injection of a viral vector (a control vector encoding GFP or an experimental vector encoding a target gene) or of cells (control cells expressing GFP or cells expressing an experimental target gene), or of tissue grafts (virally transduced or not), in a specific brain area, spinal cord, spinal ganglion, peripheral nerve, or target muscle is performed.
    a) In 80% of the experiments, this procedure is performed simultaneously with the lesioning procedure resulting in only one exposure of the animal to surgery (max 1x).
    b) In 20% of the experiments, the target expression needs to be present prior- or after the lesion has been applied resulting in a separate surgery (max 1x).
3) The animals will be sacrificed at defined time point ranging between 6 hours and 2 months after the lesion by administration of an overdose of barbiturate (I.P) or via CO2/O2 sedation followed by decapitation. Relevant tissues will be dissected out and will be processed for the molecular screen.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Parameters can be qualitative or quantitative. In the case of quantitative analysis, prior to performing an experiment we perform a power analysis (e.g. a power analysis). Many years of experience will allow us to do this in an efficient and reliable way.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mice: genetically modified and wild type; The mice are obtained from our own breedings or from a commercial licensed breeder.
Mice (adult): 240.
Rat (adult): 240.
The estimate of the total number of animals is primarily based on our experience over the past 5 years. This number of experiments and therefore the number of animals is dependent upon the number of involved researchers which is strongly dependent on the available funding. However, a general estimate for the total number of rats and mice is as follows. A typical experiment will consist of 4 groups: a control group and 3 groups at 3 post-lesion time points. Each group consists on average of 6 animals. Total number of animals for typical experiment is 24. We expect to do 2 of these experiments per year on mice (48 mice/year) and 2 of these experiments on rats (48 rats/year)which results in a total of approximately 240 mice and 240 rats over a period of 5 years.
Mice will be used when we want to measure gene and/or protein expression differences in a situation where we want to compare genetically modified (e.g. a knock-out for a target) versus wild type mice. Rats will be used for all other experiments since the size of rats allows more precise surgical lesion procedures and viral vector injections.
Importantly, before we start our experiments we will write an application to the IvD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe in full detail the experimental design, argue the number of animals in the experiments, describe human endpoints, alternatives, and the nature of discomfort. Experiments will only be started upon IvD approval.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. Testing of the intervention-techniques will be performed *in-silico* and *in-vitro* as much as possible prior to performing an animal experiment. However, to fully understand and study the proposed mechanisms/targets in the context of neurodegeneration/degeneration, animal studies are necessary because of the complexity of the processes occurring following a lesion.
These studies are worldwide conducted in both rat and mouse, making translation and extrapolation of data between the different research-groups worldwide feasible.
Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. On basis of our previous work and experience and explorative pilot experiments, statistical analysis can be performed to determine the

minimum number of animals needed to obtain scientific valuable data.
In the case when transgenic mice are used; presence of an existing mouse line is checked, and/or an attempt is made to obtain the target transgenic mouse in as little breeding steps as possible, reducing breeding time and animals. In addition, mice with inducible alleles will be used whenever possible, resulting in a normal phenotype until induction with tamoxifen.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anaesthesia and analgesia. Close postoperative monitoring will be performed and humane endpoints applied when indicated. All available resources to reduce pain, fear or suffering will be employed.
Procedures will only be performed by competent personnel, as mandatory.
Adverse environmental effects are not present.
All rats and mice will be socially housed with the appropriate environmental enrichment under strict DM2 (if a viral vector is injected) conditions, or at DM1 (if transgenic mice are used).

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed procedures are just fundamental research, it does not consist of legally required research.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

analgesia is applied prophylactic and when indicated.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

As a result of the applied loss of innervation/ neurodegeneration, a behavioral response consisting of excessive licking and biting at the affected area might occur. In time this can lead to tissue damage (autotomy). In our experience this behavior occurs mainly between 2-8 weeks post lesion and is *not* present in all types of lesions. Spinal cord and dorsal root lesions are the types of lesions where this occurs most frequently.
It is expected that in such a specific experiment 2-5% of all animals will be experiencing these adverse effect at various degrees.
All animals will be frequently monitored for possible side effects.
Animals exhibiting any unexpected phenotype resulting in constitutional discomfort will be killed within a day.

Explain why these effects may emerge.

The precise mechanism behind autotomy is still largely unknown. This behaviour only occurs in an denervated area/limb and is in the majority of cases transient.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Animals will be monitored daily and if possible treatment will be initiated (topically or systemic).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% weight loss compared to pre-operative bodyweight), abnormal/unexpected behaviour and/or posture, general signs of illness and/or discomfort. Autotomy. The animals will never experience more than moderate discomfort.

Indicate the likely incidence.

Expected 2-5 % within time frame of specific experiment in 33% of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Moderate in 100%. In most of the cases this discomfort is transient (1-2 days).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The rats or mice will be killed for histological analysis.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	NVWA 80101
1.2	Provide the name of the licenced establishment.	Netherlands Institute for Neuroscience
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: # 2 Type of animal procedure: Sacrifice of embryos of rats or mice or of adult rats or mice to obtain tissues for cell culture and bioassays.

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This project invests in the elucidation of the fundamental cell and molecular biological mechanisms that underlie the failure of the nervous system to repair itself. This fundamental knowledge is essential to identify the key molecules (also referred to as "targets") that regulate crucial aspects (neuronal survival, neurite outgrowth, synapse formation) of degeneration, regeneration, and functional recovery.

In order to identify and screen for targets and perform a pre-screening on the function and effect of these targets prior to *in-vivo* studies, molecular screens and bioassays are performed *in-vitro* using primary cell cultures obtained from rat or mice (embryos and adults) being either TG or WT. Alternatives (i.e. cell lines) are in most instances not compatible with the research question due to altered gene expression patterns resulting in the lack - or presence- of specific receptors (for example cell lines have been proven to be insensitive to semaphorins).
Animals will be killed according to Annex IV of directive 2010/63/EU and tissues will be harvested for further culturing.
Dependent on the research question and assay performed, cells are harvested from rats or mice (WT/ TG) from embryos, pups (P1-P7) or adults. To be able to answer specific research questions, primary cells from transgenic mice that are (conditional) knockout, mutant or transgenic for genes that might be associated with the above mentioned targets are needed.
These cultures are used for:

- Bioassays (outgrowth-, migration-, viability-, repulsion-, collapse- assays)
- Immunocytochemical- or in-situ hybridisation staining
- Biochemical analysis (ELISA, FACS, Microarray, RNA/ DNA/ Protein extraction).
- Manipulation of the cells (via viral- or plasmids driven gene expression, pharmacologically) and subsequent analysis using the above mentioned techniques.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

This procedure will consist of the following steps:

- The animals will be sacrificed by administration of an overdose of barbiturate (I.P), or via CO2/O2 sedation followed by decapitation.
    - In the case of embryonic tissues: pregnant mothers will be euthanized. Embryos are quickly taken from the uterus and placed on (but not in direct contact with) melting ice water, followed by decapitation. Brains will be isolated and kept cool and tissue will be harvested for culturing.
    - In the case of pups: Pups are placed on (but not in direct contact with) melting ice water, followed by decapitation. Brains will be isolated and kept cool and tissue will be harvested for culturing.
    - In the case of adult tissues: animals will be euthanized and tissue will be harvested for culturing.
- Tissues that are harvested include, but are not limited to: Brain, meninges, peripheral nerve, spinal cord, spinal dorsal root ganglia (DRG, SCG), muscle, skin.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Quantitative analysis: prior to performing an experiment we perform statistical analysis (a power analysis) to ensure that we use the minimum number of animals per group that will be statistically sound and biological relevant.
Qualitative analysis: The number of animals is based upon our large experience in the past. This concerns knowledge about the total number of cells that can be obtained from specific tissues per animal and the expansion of these cells during passaging. Experimental design further dictates the number of cells needed. Finally, experiments are performed sequentially resulting in increasing knowledge about the variation in the target and control groups.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The estimate of the total number of animals is primarily based on our experience over the past 5 years. This number of experiments and therefore the number of animals is dependent upon the number of involved researchers which is strongly dependent on the available funding. However, in general, an estimate for the total number of rats and mice is as follows:
Rat and mice embryo's: an average of 25 pregnant mice and 25 rat pregnant mothers (litters) is needed per year to perform primary cultures and bioassays

(e.g. dorsal root ganglia) described in this protocol. One litter generally provides enough cells from any of the proposed tissues, to perform an in-vitro study (including experimental groups, positive- negative- and biological controls). For 5 years, 125 rat and 125 mice pregnant mothers, with an average of 6 to 8 pups per mother. *For 5 years that amounts to maximally 1000 rat embryos and 1000 mouse embryos (and the 125 rat and 125 mice pregnant mothers).*

Early post-natal rats and mice (P1 to P7): an average of 75 rat pups and 75 mouse pups is required per year to perform primary cultures and bioassays using post-natal cells (e.g. cerebellar granule cells, cortical neurons). *For 5 years a total of 375 rats and 375 mice pups are required.*

Adult rats and mice: an average of 20 adult rats and 20 adult mice is necessary to set up primary cultures and bioassays using adult neurons (mostly dorsal root ganglion neurons). *For 5 years a total of 100 adult rats and 100 adult mice are required.*

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. Testing of the intervention-techniques will be performed *in-silico* and *in-vitro* as much as possible prior to performing an animal experiment.
In case an alternative (i.e. a cell line) is available that is applicable and will answer the research question, these are the primary choice.
Optimal use is made from each animal killed by harvesting the maximum amount of tissue from different organs.
These studies are conducted in both rat and mouse worldwide, making translation and extrapolation of data between research-groups more feasible.
Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. On basis of this previous work and experience, statistical analysis can be performed to determine the minimal number cells/ culture dishes and thus, the minimal number of animals needed to obtain valuable data.
To reduce inter- and intra-assay variability we will only use well-established reagents and protocols during the in-vitro procedures.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All procedures resulting in animal suffering or pain will be performed under adequate anaesthesia. All available resources to reduce pain, fear or suffering will be employed.
Experiments will be done sequentially. Whenever possible small scaled pilot studies will be performed with the minimal number of animals.
Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.
All rats and mice will be housed under strict DM1 and SPF regulations.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed procedures are just fundamental research, it does not consist of legally required research.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☒ No > Continue with question I.

☐ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☐ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

No other adverse effects are expected. Animals will experience normal housing conditions without additional handling untill they are killed.

Explain why these effects may emerge.

Not applicable

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Not applicable

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☒ No > Continue with question K.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

Expected 0% within time frame of the experiment

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

mild 100%

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	NVWA 80101
1.2	Provide the name of the licenced establishment.	Netherlands Institute for Neuroscience
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: #3 Type of animal procedure: Injury models (functional and histological analysis): Injection of cells or viral vectors in lesioned animals.

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Our aim is to determine whether our identified target molecules and intervention methods (i.e. viral vector or cell-mediated gene delivery of a target, by using transgenic mice with overexpression or knock-down of a target) will improve both anatomical and functional recovery following a lesion to the brain, spinal cord, or peripheral nerve. Therefore, following a lesion we will perform several non-invasive function tests and/or an electrophysiological test on each

animal at several time points after induction of the lesion and the therapeutic intervention. This will provide insight in the full spectrum of the induced functional deficit and the degree of subsequent recovery of function per animal in time.
The brain, spinal cord or peripheral nerve lesions will be made by means of well-established surgical procedures. These lesion types are chosen because they represent clinically relevant injuries in which we can test the efficacy of a specific intervention. In different parts of the CNS different processes underlie degeneration and regeneration and it is therefore important to study different parts of the CNS (brain, spinal cord, spinal ganglion, peripheral nerve) and innervated target muscles.
The cells or viral vectors will be injected via small needles in the area of study.
Activation or inhibition of transgenes encoded in the viral vectors or transgene mouse via pharmaceuticals (e.g. doxycycline, tamoxifen) might be necessary to regulate transgene expression in time.
Intravenous application of a substrate (e.g. luciferine) might be necessary to perform an imaging study and visualize viral vector mediated expression of the reporter gene luciferase.
Assessment of recovery of function is obtained by performing multiple function tests at multiple time points.
Animals might be injected under anaesthesia with an antero/retrograde tracer prior to killing the animals to allow histological assessment of regeneration/sprouting process at the intermediate or the final stages of recovery.
At the end of the experiments in all cases the animals will be killed and tissues will be harvested for further analysis, allowing direct correlation between the individual degree of function recovery and histological parameters. These tissues can be subjected to: histological sectioning followed by immunohistochemical- or in-situ hybridisation staining or biochemical analysis (ELISA, FACS, Microarray, RNA/ DNA/ Protein extraction).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

This procedure will consist of the following steps:

1) *Pre-training and baseline measurement of the animals using behavioural function tests*. Acclimatization of the animals to both the handling and testing-environment will result in reliable baseline data with little variation. This step involves:
    a. Pre-training on our non-invasive function tests performed on freely moving animals to obtain baseline values *(max: 1x exposure/ test/ day, for 2 weeks).* In general, animals performing one or more non-invasive function tests including, for example: 1) cross a beam, rope or narrow corridor, walking from platform to platform without being forced. 2) Grip a horizontal bar and pull after which the maximum applied force is measured. 3) Walk in an open field or cylinder while being observed by the researcher who is scoring naturally behaviour 'events'. Dependent on the aim of the experiment the relevant test(s) and variants thereof will be selected and applied.
2) Baseline measurement of electrophysiological (CMAP) measurement under adequate anaesthesia. *(max: 2x)*
3) *Application of a lesion to the brain, spinal cord or peripheral nerve*. Under adequate anaesthesia and postoperative analgesia, the skull, spinal cord or peripheral nerve is exposed and a unilateral or bilateral lesion is made by means of damaging of a nerve tract.
    a. *Brain*: injection of a toxin systemically (I.P.) or intracranially, or by means of mechanical injury with microscissors *(max 1x).*
    b. *Spinal cord*: At cervical or thoracic or lumbar level, access to the spinal cord is gained and a uni- or bilateral lesion of the rubrospinal tract, corticospinal tract, dorsal column or dorsolateral columns is performed by means of microscissors *(max 1x).*
    c. *Peripheral nerve*: After gaining access to the peripheral nerve lesion site, unilateral crush or transection or spinal root avulsion is performed. *(max 1x).*
4) *Viral vector or cell-mediated gene delivery of a target.* Under adequate anaesthesia and postoperative analgesia a (stereotaxic) injection of a viral vector (an control vector encoding GFP or 'repair experiments' with an experimental vector encoding a target gene) or of cells (control cells expressing GFP or cells expressing an experimental target gene), or of tissue grafts (virally transduced or not), in a specific brain area, spinal cord, spinal ganglion, peripheral nerve, or target muscle is performed.
    a. In 80% of the experiments, the injection of the viral vector is performed simultaneously with the lesioning procedure resulting in only one

exposure of the animal to surgery *(max 1x).*
   b. In 20% of the experiments, the target expression needs to be present prior- or after the lesion has been applied resulting in a separate surgery (*max 1x*).
5) Dependent on the viral vector system or transgenic mouse used, administration of transgene-inducing agent or pharmaceuticals are needed continuously or alternating, as follows:
   a. Enteral: diet supplemented with doxycyclin (during certain periods of the experiment)
   b. Parenteral: intravenous, intramuscular, intraperitoneal injection of luciferine a substrate for luciferase a reporter gene used to monitor the activity of some vectors. Subsequent imaging to visualize viral vector mediated expression of the reporter gene luciferase is performed in the IVIS under isoflurane anaesthesia. These measurements generally take 5 minutes, but maximally 30 minutes (*max 1x/wk, max 24x*).
   c. Parenteral: I.P. injection of tamoxifen (*max 3x*).
6) *Testing the efficacy of the treatment by assessment of recovery of function:* The initial loss and gradual gain of function occurs over a period of maximal 12 months and will be evaluated by performing a subset of the function tests as described in (1). The frequency of testing is:
   a. Early following the lesion (first 4-8 weeks), (bi-) weekly tests need to be performed in order to evaluate the dynamics of this initial recovery phase.
   b. After 4-8 weeks, gain of function will start to level off and a weekly- to bimonthly testing frequency up to the final 12th month is sufficient.
7) *Testing the efficacy of the treatment by assessment of anatomical parameters*:
   a. *Retrograde tracing to histologically visualize treatment efficacy prior to sacrifice.* Administration of an antero- or retrograde tracer by:
      1. Surgically exposing the peripheral nerve followed by tracer application under adequate anesthesia and analgesia (*max. 1x*)
      2. Surgically exposing the skull/spinal cord followed by intracranial or intraspinal tracer injection under adequate anesthesia and analgesia (*max.1 x*)
   b. Perfusion fixation of animals at specific time points after the lesion by sacrificing the animals by administration of an overdose of barbiturate (I.P) followed by transcardial perfusion/fixation, or via CO2/O2 sedation followed by dissection of the relevant tissues for extensive histological analysis of the tissue response to the treatment.
8) (Optional) Withdrawal of blood samples without anesthesia in the mouse, or under adequate anaesthesia in the rat. (*max 5x*).
9) (Optional) Imaging by a light source (luminescence): the intravenous or intraperitoneal administration of luciferin under isoflurane anesthesia for a period of max 10 minutes (*max 2x week up over a period of maximally 12 months*).
10) The animals will be sacrificed by administration of an overdose of barbiturate (I.P) followed by transcardial perfusion/fixation, or via CO2/O2 sedation followed by decapitation.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Quantitative analysis: prior to performing an experiment we perform statistical analysis (a power analysis) to ensure that we use the minimum number of animals per group that will be statistically sound and biological relevant.
Qualitative analysis (most of our experiments): the number is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially in order to ensure that we will use the minimum number of rats or mice per group that is informative resulting in scientifically sound conclusions.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mice: genetically modified and wild type; The mice are obtained from our own breedings or from a commercial licensed breeder.
Mice (adult): 750.

Rat (adult): 900. The rats are obtained from a commercial licensed breeder

The estimate of the total number of animals is primarily based on our experience over the past 5 years. This number of experiment and therefore the number of animals is dependent upon the number of involved researchers which is strongly dependent on the available funding. However, in general, an estimate for the total number of rats and mice is as follows: Function studies contain an average of 60 rats (containing experimental groups, positive- negative- and biological controls). In average 3 of these studies are performed each year, resulting in a total of 900 rats (5 year x 3 studies x n=60). The same is true for mouse function studies, resulting in 900 mice total.

Before we start our experiments we will write an application to the IvD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe in full detail the experimental design, argue the number of animals in the experiments, describe (i.a.) human endpoints, alternatives, nature of discomfort. Experiments will only be started upon IvD approval.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. Testing of the intervention-techniques will be performed *in-silico* and *in-vitro* as much as possible prior to performing an animal experiment. However, to fully understand and study the proposed mechanisms/targets, animal studies are necessary as the complexity of the processes occurring following a lesion can only be achieved in a complete organism.

These studies are conducted in both rat and mouse worldwide, making translation and extrapolation of data between research-groups more feasible. Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. On basis of this previous work and experience, statistical analysis can be performed to determine the maximum number of animals needed to obtain valuable data.

In the case when transgenic mice are used; presence of an existing mouse line is checked, and/or an attempt is made to obtain the target transgenic mouse in as little breeding steps as possible, reducing breeding time and number of animals. In addition, mice with inducible alleles will be used when possible, resulting in a normal phenotype until induction.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anaesthesia and analgesia. Close postoperative monitoring will be performed and humane endpoints applied. All available resources to reduce pain, fear or suffering will be employed.

Procedures will only be performed by competent personnel, as mandatory.
Adverse environmental effects are not present.
All rats and mice will be socially housed and provided with tools for environmental enrichment. For some periods during the experiments they are housed under strict DM2, DM1 regulations (no discomfort consequences).

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed experiments are not carried out as a result of legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

anaesthesia and analgesia is used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

As a result of the applied loss of innervation/ neurodegeneration, autotomy, a response consisting of excessive licking and biting at the affected area, might occur. In time this can lead to tissue damage. In our experience this behavior occurs mainly between 2-8 weeks post lesion and is *not* present in all types of lesions. Spinal cord and dorsal root lesions are the types of lesions were this occurs most frequently.
It is expected that in 33% of the experiments 2-5% of all animals will be experiencing these adverse effects to different degrees.
All animals will be frequently monitored for possible side effects.
Animals exhibiting any unexpected phenotype will be killed within a day.

Explain why these effects may emerge.

The precise mechanism behind autotomy is still largely unknown. This behaviour only occurs in an denervated area/limb and is in the majority of cases transient.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Animals will be monitored daily and if possible treatment will be initiated (topically or systemic).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% weight loss compared to pre-operative bodyweight), abnormal/unexpected behaviour and/or posture, general signs of illness and/or discomfort. Autotomy. The discomfort will never be more that moderate.

Indicate the likely incidence.

Expected 2-5 % within time frame of the experiment.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Moderate 100%. In most of the cases this discomfort is transient (1-2 days).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

the rats or mice will be killed for histological analysis.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	NVWA80101
1.2	Provide the name of the licenced establishment.	Netherlands Institute for Neuroscience
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: # 4 Type of animal procedure: Testing of the quality of viral vector batches

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

A primary aim of our research is to unravel new mechanisms that govern or hamper neuroregeneration or neuroplasticity. A key approach that we use to test the efficacy of a specific target molecule is viral vector-mediated gene transfer to neurons or glia cells. Although the viral vector technique has become more and more a standard technique, the generation of new viral vectors with potentially improved performance is an ongoing endeavor. For instance, of one of the most used viral vectors (adeno-associated viral vectors - AAV) an increasing number of variants ("serotypes") with specific cellular transduction profiles have

become available. Moreover we are currently developing vectors with regulatable transgene expression. Therefore it is necessary to have a protocol in place that allows testing the performance of newly generated vectors in small scale prior to their use in large animal experiments. A new vector batch that needs to be tested will only be tested in the tissue for which it is intended to be used later in the large experiment in which its efficacy is tested. Therefore this test protocol includes injections in the brain, spinal cord, peripheral nerve, and muscle without surgically inflicted damage.
Injection of a viral vector in the brain, spinal cord or the peripheral nerve or muscle will be performed by means of well-established (stereotactic) injection procedures. The typical primary outcome parameters that will be studied are: the transduction efficiency (number of cells transduced), the level of transgene expression per cell, the spread and cell type specificity obtained with a viral vector and, in the case of a regulatable viral vector, the inducibility and subsequent silencing of transgene expression.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

This procedure can consist of the following steps:

1) Injection of a vector in the brain, spinal cord or the peripheral nerve or muscle. Dependent on the specific aim of the project for which the vector is generated the vector will be tested in the tissue of interest for that project (max. 1 x) For most vectors is will be sufficient to test the performance on a limited number of port-injection times, e.g. 2 weeks and 4 weeks. For other tests of viral vectors more elaborate testing will be required, e.g. for regulatable vectors where a gene is turned on and off.
2) Specific virus batch-dependent situations:
3) Dependent on the viral vector system or transgenic mouse used, administration of transgene-inducing agent or pharmaceuticals are needed continuously or alternating, as follows:
    a. Enteral: diet supplemented with doxycyclin (during certain periods of the experiment)
    b. Parenteral: intravenous, intramuscular, intraperitoneal injection of luciferine a substrate for luciferase a reporter gene used to monitor the activity of some vectors. Subsequent imaging to visualize viral vector mediated expression of the reporter gene luciferase is performed in the IVIS under isoflurane anaesthesia. These measurements generally take 5 minutes, but maximally 30 minutes (*max 1x/wk, max 24x*).
    c. Parenteral: I.P. injection of tamoxifen (*max 3x*).

    Vectors encoding the recombinase Cre will be tested in genetically modified mice that carry a floxed gene of interest or a floxed reporter gene which requires i.p injection of tamoxifen
4) At the end of the experiment the animals will be sacrificed by administration of an overdose of barbiturate (I.P) followed by transcardial perfusion/fixation, or via CO2/O2 sedation followed by decapitation. The expression of the transgene will be studied by histological analysis of the tissue or by biochemical analysis, e.g. and ELISA for GFP or GDNF.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

We will not use statistical method to determine the number of animals in this particular procedure because the aim of this procedure is not to compare groups with different treatments. Based on previous experiments we know that an N=4 per group is normally sufficient to determine whether a viral vector batch works well or not. The N=4 is based on the following consideration: our injection techniques are well-established, however, occasionally we lose an animal because the injections does not go optimal. With an N=4 we always have at least 3 animals in which we will be able to investigate transgene expression.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mice (adult): 180. Either commercial or wildtype/transgenic mice from our own breeding facility.
Rat (adult): 460. Commercially available.

The estimate of the total number of animals is primarily based on our experience over the past 5 years.
The number of experiments and therefore the number of animals is dependent upon the number of involved researchers which is strongly dependent on the available funding.
Adult mice. We estimate that a total number of 7 viral vector batches have to be tested in mice. We expect that 1 batch will have to be tested on 3 timepoints with 4 mice per time point. For the other 6 batches one time point would be suffcient, 4 mice per time point. Per year we need 36 mice. Total per 5 years: 180 mice. The justification of the use of mice is that we use viral vectors that express Cre, a recombinase, to knock-out a specific gene that is floxed in mice.The advantage of the use of Cre expressed via a viral vector is that this can be done in adult animals, that is in circumstances where the development of the animal has been completely normal.
Adult rats. We estimate that a total of 15 viral vector batches have to be tested in rats. Total rats per 5 years is 460. The justification of the use of rats is that our lesion and regeneration models are esthablished in rats.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The aim of this protocol is to test the performance of viral vector batches prior to their use in larger animal experiments. This avoids that batches that do not perform as required are not used in larger animal experiments. This "pre-screening" of the performance of a viral vector batch results in the reduction of the use of animals because it avoids the use of a "bad" batch in larger experiments that would fail if the pre-screen would not have been done. Pre-screening is also a form of refinement.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anaesthesia and analgesia. Close postoperative monitoring will

be performed and humane endpoints followed. All available resources to reduce pain, fear or suffering will be employed.
Procedures will only be performed by competent personnel, as mandatory.
Adverse environmental effects are not present.
All rats and mice will be socially housed with the appropriate environmental enrichment under standard and when required DM2, DM1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed experiments are not carried out as a result of legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

anaesthesia and analgesia is used

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Effects due to the performed surgery.
Effects as a consequence of the biological effects of the applied vector. No discomfort is expected.
All animals will be frequently monitored for possible side effects. Animals exhibiting any unexpected phenotype? will be killed within a day.

Explain why these effects may emerge.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Animals will be monitored daily and if possible treatment will be initiated (topically or systemic).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% weight loss compared to pre-operative bodyweight), abnormal/unexpected behaviour and/or posture, general signs of illness and/or discomfort.

Indicate the likely incidence.

Expected 2-5 % within time frame of the experiment.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Mild 100%

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The rats or mice will be killed for histological analysis.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
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1 General information

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1.2	Provide the name of the licenced establishment.	Netherlands Institute for Neuroscience
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: # 5 Type of animal procedure: Monitoring and generation of novel genetically modified mice

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

This procedure concerns the creation of genetically mice via DNA/RNA injection into oocyte, injection of genetically modified ES cells into blastocysts and/or via the CRISPR/Cas9 system. Moreover this procedure concerns the generation of crosses between mice with a floxed allel and Cre-expression mice lines in order to generate conditional null-mutant mice. As a consequence of this advanced breeding procedure mice may only have a gene deletion in a particular neuron or glia cell.

Welfare assessment of the novel mouse models will be performed according to the guidelines of the new EU directive. New transgenic lines and/or KO lines generated via classical methods and/or novel combinations of these aforementioned lines will be monitored for 2 generations to determine the absence or presence of mice with a deviant or hampered phenotype. Since whole body (compound) knock-outs will now be mostly replaced by cell specific knock-outs we expect that phenotyes will display considerable less adverse effects.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Generation of the mice according to classical methods:
1) Superovulation of donor mice.
   a) Administration of gonadotropin's (2 times) by subcutaneous or intraperitoneal injections followed by mating.
   b) Animals will be killed for the isolation of early (usually two or four cell stage) embryos.

2) Embryo recipients.
   a) Recipients for embryo transfer will be rendered pseudo pregnant by mating with a sterile (vasectomized) male.
   b) Genetically modified embryos will be implanted surgically or non-surgically into the reproductive tract.
   c) Embryo recipients, not as part of an experiment, will be killed after weaning of the pups at three weeks of age.

3) Weaned pups at 3 weeks of age: Tissue sampling for genotyping and/or identification via tail and earcut, respectively, under anesthesia (isoflurane).

Animals are killed by O2/CO2 method.

Welfare assessment:
Daily checks of the welfare of the mice on several common parameters (overall appearance, size, confirmation and growth, coat condition, behavior, clinical signs, relative size and numbers) as has been described in the Directive 2010/63/EU: corrigendum of 24 Jan. 2013

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

For these type of experiments statistical analysis is not performed since the purpose of the experiment is not to compare groups but to create viable novel mice lines for follow-up experiments. All techniques are state of the art and have been shown to be effective in generating GM mice with a smallest number of mice possible.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mouse; Mus musculus: genetically modified and wild type adult mice. All mice are derived from the NIN, an establishment licensed by the NVWA, or from a registered commercial company.

For generation of GM mice we expect, based on our extensive experience, to generate max. 15 new lines over the next 5 years. For the creation of a new GM mouse lines we will use on average max. 150 mice (according to the besluit biotechnologie). Based on these numbers in total a maximum of 2250 mice will be required.

Welfare assesment: we expect to generate over the next 5 years 15 new GM lines for which we have to perform the welfare assesment. For 2 generations, 7 males and 7 females control and GM mice. We therfore need in total: 15 (new (compound) lines) * 2 (generation) * 28 ((7 male +7 female = 14 GM mice +

(7 male + 7 female = 14 control mice)) = 840 mice for the welfare assessment.

Taken together within the context of this procedure we need 2250 + 840 mice = 3090 mice

A large portion of the newly generated GG mice will be floxed mice, which have no phenotype by definition, and which are not part of the welfare assesment protocol. We will not breed new GM mice showing a hampered phenotype.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

☒ Yes > Explain why re-use is considered acceptable for this animal procedure.

- (Vasectomized) males will also be used by the other groups of the NIN if required for their experiments, thereby reducing the number of (vasectomized) males used for the generation of GM mice.
  Mice used for welfare assessment, might be used for experiments described in procedures 3.4.4.1, 3.4.4.2 and 3.4.4.3.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

We start the generation of a new GM mouse line only after we are convinced that based on molecular screens in human tissue or animals (procedure 3.4.4.1), and in vitro experiments (procedure 3.4.4.2) the creation of a new line is essential for in vivo functional and mechanistic studies. Animal studies are essential unavoidable if we want to obtain comprehensive knowledge on the function of specific genes in processes of neuroregeneration and plasticity. The CRISPR/Cas9 system allows us, if required, to genetically modify multiple (that is up to 5 different) genes in a single experiments. This may strongly reduce the number of mice used for the generation and/or breeding of these GM mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under well-controlled DM1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed procedures are just fundamental research, it does not consist of legally required research.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

We do not expect to find additional adverse effect. This is the direct result of how we create our constructs for the generation of GM mice.

Explain why these effects may emerge.

We do not expect to find other adverse effect.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of our mice; immediate action will be taken immediately if unexpectedly any adverse effect will become visible.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☒ No > Continue with question K.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Vasectomized males: moderate
Donors: moderate 100%
Foster mothers: moderate 100%
GM mice: no to mild 100% (welfare assessment).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The donor females will be killed as part of the experiments.
The foster females will be killed after the experiment (at the stage of weaning of the pups).

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD801002015104

Bijlagen
2

Datum 22-05-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 22 mei 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD801002015104. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. Zodra uw aanvraag compleet is, ontvangt u binnen veertig werkdagen een beslissing op uw aanvraag. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan wordt uw aanvraag buiten behandeling gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	80100
Naam instelling of organisatie:	Kon. Ned. Academie van Wetenschappen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	54667089
Postbus:	19121
Postcode en plaats:	1100 GC AMSTERDAM
IBAN:	NLDEUT056900054
Tenaamstelling van het rekeningnummer:	Nederlands Herseninstituut

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	[redacted]
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 juli 2015
Geplande einddatum:	1 juli 2020
Titel project:	Developing strategies to promote repair or plasticity of the central and peripheral
Titel niet-technische samenvatting:	Ontwikkeling van strategieën om de regeneratie van zenuwweefsel te bevorderen
Naam DEC:	DEC KNAW
Postadres DEC:	[redacted] Amsterdam
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 741,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] DEC-advies [x] er zijn in totaal 5 appendixen

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Amsterdam
Datum:	18 mei 2015

Centrale Commissie Dierproeven

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Bijlagen
2

Datum 22-05-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 22 mei 2015
Vervaldatum: 21 juni 2015
Factuurnummer: 201570104

Omschrijving	**Bedrag**
Betaling leges projectvegrunning dierproeven Betreft aanvraag AVD801002015104	€ 741,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD801002015104

Datum
Betreft Vervolg Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Op 22 mei 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project Developing strategies to promote repair or plasticity of the central and peripheral met aanvraagnummer AVD801002015104. Uw aanvraag wordt in behandeling genomen. In deze brief leest u wanneer u een beslissing kunt verwachten.

Wanneer een beslissing
Wij nemen uiterlijk 17 juni 2015 een beslissing. Als wij nog informatie nodig hebben, kan dit later worden. Voor een complexe aanvraag staat een langere termijn. In beide gevallen ontvangt u daarover bericht. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

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Datum
Betreft Vervolg Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Op 22 mei 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project Developing strategies to promote repair or plasticity of the central and peripheral met aanvraagnummer AVD801002015104.

DEC advies gevraagd
Uw aanvraag is naar DEC Kon. Ned. Academie van Wetenschappen gestuurd. Zij zal hierover advies aan de CCD uitbrengen. Als de DEC vragen heeft, zal zij contact met u opnemen.
Uw aanvraag wordt door een andere dan de door u aangegeven DEC van een advies voorzien. nvt

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de bijbehorende toelichting, waarin elke stap in het beoordelingsproces wordt toegelicht

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD/801002015104
2. Titel van het project: Developing strategies to promote repair or plasticity of the central and peripheral nervous system.
3. Titel van de NTS: Ontwikkeling van strategieën om de regeneratie van zenuwweefsel te bevorderen.
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 17-04-2015
   - ✓ aanvraag compleet: 30-04-2015
   - ✓ in vergadering besproken: 23-04-2015
   - ✓ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en): n.v.t.
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ✓ advies aan CCD: 22-05-2015
7. Eventueel horen van aanvrager
   - Datum: n.v.t.
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
8. Correspondentie met de aanvrager:
   - Datum 24-04-2015
   - Strekking: completering van de aanvraag
   - Datum antwoord 20-05-2015
   - Strekking van de antwoorden: de aanvraag is gecompleteerd
9. Eventuele adviezen door experts (niet lid van de DEC): geen

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig. Het omvat dierproeven in de zin der wet.

2. De aanvraag betreft een nieuwe aanvraag. Er is enige overlap met een aantal al van een positief advies voorziene DEC-protocollen.
3. De DEC is competent om over deze projectvergunningsaanvraag te adviseren. De benodigde expertise op dit wetenschappelijk terrein is aanwezig binnen de DEC. Geen van de DEC-leden is betrokken bij het betreffende project.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering: n.v.t.

C. Beoordeling (inhoud):

1. Het project is wetenschappelijk verantwoord.

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

3. De doelstelling, in relatie tot de uitvoering, is helder omschreven; te weten het verkrijgen van fundamenteel wetenschappelijke inzichten in 1) de biologische mechanismen van het afsterven van zenuwcellen en de biologische mechanismen van zenuwweefselregeneratie na het optreden van schade en 2) de toepasbaarheid van die kennis in de ontwikkeling van therapeutische strategieën om het verlies van zenuwcellen te voorkomen of om de uitgroei van beschadigde zenuwuitlopers te stimuleren. Op termijn kunnen de resultaten leiden tot nieuwe behandelingsmethoden voor de humane patiënt met zenuwschade.

   Het fundamenteel wetenschappelijke belang acht de DEC substantieel. Het verkrijgen van wetenschappelijke kennis van de processen en factoren die ten grondslag liggen aan regeneratie van zenuwweefsel (waaronder het herstel van zenuwceluitlopers en het voorkomen van de celdood van zenuwcellen) is essentieel voor het ontwikkelen van nieuwe therapeutische strategieën. Inzichten in het gebruik van gentherapie met behulp van virale vectoren om de geïdentificeerde factoren te kunnen inbrengen op de juiste plaats in het geval van zenuwschade is naar de mening van de DEC van substantieel belang. Het project dient een belangrijk maatschappelijk belang, gezien de grote groep patiënten met zenuwweefselschade.

4. De gekozen strategie en experimentele aanpak in combinatie met de infrastructuur op het Nederlands Herseninstituut en de expertise van de betrokken onderzoeksgroep bieden een realistisch uitzicht op het behalen van de beoogde doelstellingen binnen gevraagde looptijd van het project. Het project bouwt voort op een langlopende lijn van onderzoek van een grote groep onderzoekers. Over de afgelopen jaren zijn met een vergelijkbare strategie en aanpak belangrijke wetenschappelijk resultaten behaald, resulterend in vele publicaties in vooraanstaande tijdschriften. Het onderzoek wordt financieel gesteund door verschillende onafhankelijke subsidiegevers. Er zijn internationale samenwerkingsverbanden en er zijn sterke relaties met de kliniek die een

vertaling van de bevindingen van het onderzoek naar de kliniek zullen vergemakkelijken.

5. Alle dieren worden gefokt voor het gebruik in dierproeven, er is geen sprake van afwijkende huisvesting en/of hergebruik. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit het wild. De toegepaste methoden voor anesthesie/euthanasie zijn conform de Richtlijn.

6. Het cumulatieve ongerief gepaard gaand met de dierproeven, zoals beschreven in de vijf verschillende type dierproeven, is naar inschatting van de DEC licht (Type dierproef 2, 4 en 5) of matig (Type dierproef 1 en 3) ongerief. In het merendeel van de gevallen met matig ongerief is de duur van het ongerief beperkt tot 1-2 dagen en is er een beperkt risico op onbedoelde bijwerkingen in de vorm van autotomie. Deze inschatting van de DEC is in overeenstemming met het niveau van cumulatief ongerief zoals dat is geclassificeerd door de onderzoekers. Dit is gebaseerd op hun ruime ervaring met de gebruikte modellen in vergelijkbare dierproeven.

7. Binnen het project wordt maximaal gebruik gemaakt van methoden die de voorgestelde dierproeven geheel of gedeeltelijk **vervangen.** Een belangrijk onderdeel van de experimentele strategie is de gefaseerde opzet. In de eerste fase, voorafgaand aan de dierproeven, vindt een uitgebreide screening plaats met weefsel afkomstig van patiënten en van de Nederlandse Hersenbank. Na deze fase zijn er go/no-go-beslissingsmomenten, voordat tot het uitvoeren van bioassays wordt besloten.
Nieuwe inzichten in de processen die zenuwweefselherstel reguleren kunnen op dit moment alleen maar verkregen worden in een intact organisme. Deze processen, waarbij verschillende typen cellen betrokken zijn binnen een gecompliceerde anatomische context, zijn zeer complex en kunnen niet met cellijnen worden bestudeerd. Naar het oordeel van de DEC zijn er geen alternatieven beschikbaar voor het voorgestelde gebruik van intacte dieren om te doelstelling van dit project te realiseren.

8. In het project wordt optimaal tegemoet gekomen aan de vereisten van **vermindering** van dierproeven. De onderzoeksgroep heeft een jarenlange ervaring opgebouwd met dit soort experimenten en door een veelal gefaseerde opzet wordt per experiment niet meer dan het minimaal benodigde aantal dieren ingezet. Voorafgaand aan de kwantitatieve experimenten wordt op basis van literatuurgegevens, eigen historische data of een specifiek hiertoe uitgevoerd pilot experiment de groepsgrootte bepaald. Technieken en procedures worden zorgvuldig toegepast. Het aantal te gebruiken dieren is realistisch geschat.
Voor de bioassays zijn op beperkte schaal proefdieren nodig (met licht ongerief) maar de uitkomsten van deze proeven leiden tot de selectie van factoren met een duidelijk effect en hiermee wordt het aantal dierproeven met

zenuwschademodellen (met matig ongerief) verminderd en wordt de kans op het verkrijgen wetenschappelijk relevante resultaten verhoogd.
Voor het genereren van genetisch gemodificeerde diermodellen waarin de gentherapie experimenten worden gedaan zijn naar verhouding veel dieren nodig. Ook ontstaat daarbij onvermijdelijk een overschot van dieren die wel gefokt worden, maar niet worden gebruikt in het onderzoek. De commissie acht dat aanvaardbaar in het licht van het feit dat de gentherapie experimenten, zowel in methodologisch opzicht (onderzoek naar de functie van een gen in beschadigd zenuwweefsel), als mogelijke toekomstige klinische behandeling, van essentieel belang zijn voor dit project.

9. De uitvoering van het project is in overeenstemming met de vereisten van **verfijning** van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.
Bij de opzet wordt rekening gehouden met dierenwelzijn en wel op de volgende manieren: 1) het gebruik van adequate anesthesie en analgesie waar nodig, 2) een intensieve monitoring van de proefdieren na de inductie van zenuwweefselschade, 3) het gebruik van weefselspecifieke genetisch gemodificeerde muizen, 4) een monitoring op het optreden van onverwacht constitutioneel ongerief van nieuwe gecreëerde genotypes.
Er is geen sprake van belangwekkende milieueffecten.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en is geformuleerd in begrijpelijke taal. De NTS voldoet daarmee aan de eisen zoals gesteld in artikel 10.a.1.7 van de Wod.

D. Ethische afweging

De centrale vraag voor de ethische afweging is of het belang van het doel van dit project opweegt tegen het ongerief dat de dieren ondergaan (geclassificeerd als licht of matig).Het doel van het project is het verkrijgen van fundamenteel wetenschappelijke inzichten in: 1) de biologische oorzaken van het afsterven van zenuwcellen en het (vrijwel volledig) ontbreken van zenuwweefselregeneratie en 2) de toepasbaarheid van die kennis in de ontwikkeling van een strategie (in het bijzonder toepassing van virale gentherapie) om het verlies van zenuwcellen te voorkomen of om de uitgroei van beschadigde zenuwuitlopers te stimuleren.
Het onderzoek is primair fundamenteel wetenschappelijk van karakter. De verwachting is dat de resultaten op den duur kunnen bijdragen aan nieuwe therapieën om de gevolgen van zenuwweefselschade voor patiënten te verminderen. Voor een grote groep patiënten met diverse typen zenuwweefselschade van is het van aanzienlijk belang dat er uitzicht ontstaat op nieuwe therapieën die de kwaliteit van hun leven aanmerkelijk zal kunnen verhogen.

Het fundamenteel wetenschappelijke onderzoek in dit project is van aangetoonde en excellente kwaliteit. De onderzoeksgroep beschikt over ruime ervaring met de gekozen onderzoeksstrategie en met de vijf beschreven type dierproeven.

De classificatie van het ongerief van de dieren in de verschillende typen dierproeven is licht of matig. De intrinsieke waarde van het dier wordt door de laesiemodellen in lichte mate aangetast wanneer de toegebrachte schade resulteert in een lichte verlamming. Bij de het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald.

De DEC is van mening dat de resultaten van dierproeven zullen bijdragen aan het behalen van het geformuleerde doel en schat de kans op het realiseren van de fundamenteel wetenschappelijke doelstellingen in als hoog. Het project is uit wetenschappelijk oogpunt verantwoord. De verkregen fundamenteel wetenschappelijke kennis is essentieel om te komen tot een toepassing van regeneratieve (gen)therapieën in patiënten met zenuwweefselschade. De verwachting is dat deze nieuwe therapieën effectiever zullen zijn dan de huidige therapieën. Het gaat om een grote groep patiënten met uiteenlopende, op dit moment nog slecht behandelbare, aandoeningen. Het maatschappelijk belang is daarom groot.
De DEC komt tot de conclusie dat de doeleinden van het project het voorgestelde gebruik van de proefdieren en het daarmee samenhangende ongerief van de proefdieren rechtvaardigt.

E. Advies

1. Advies aan de CCD
   - ✓ **De DEC adviseert de vergunning te verlenen**
2. Het uitgebrachte advies is gebaseerd op consensus.
3. Er zijn geen knelpunten of dilemma’s gesignaleerd tijdens het beoordelen van de aanvraag of het formuleren van het advies.

NEDERLANDS HERSENINSTITUUT
Master the mind

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for Neuroscience

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Aan de CCD
t.a.v. [redacted]
Postbus 20401
2500 EK Den Haag

Amsterdam, 12 juni, 2015

Geachte mevrouw [redacted]

Hierbij beantwoord ik uw vragen betreffende het project “Developing strategies to promote repair or plasticity of the central and peripheral nervous system” met nummer AVD 801002015104 zoals gesteld in uw e-mails van 5 juni en 11 juni j.l.

Uw vraag (5 juni): In de beschrijving dierproeven 3.4.4.3; Injury models: Bij punt B beschrijft u in de eerste alinea een aantal van 750 genetisch gemodificeerde of WT muizen. Verderop in de tekst berekend u voor de injury models 900 ratten en 900 muizen. Het wordt uit de tekst niet helemaal helder of het totale aantal 750 GGO muizen + 900 ratten + 900 muizen is. Kunt u dit toelichten en als nodig in de tekst aanpassen?

Mijn antwoord: er zijn voor dit type dierproef in totaal 900 muizen en 900 ratten nodig. De berekening van het aantal muizen op 750 berust op een (reken)fout. In de tekst van 3.4.4.3 is voor beide diersoorten nu 900 aangehouden. In de NTS stond het juiste aantal dieren aangegeven. Bijgevoegd is een tabel met de dieren aantallen per appendix uitgesplitst naar diersoort en verwacht ongerief (Bijlage Table of Groups). Wij verzoeken u deze tabel als bijlage bij de PVA op te nemen in het dossier.

Uw vraag (5 juni): In beschrijving dierproeven 3.4.4.4; testing of the quality of viral vector batches, zit een tekstuele inconsequentie: bij punt A beschrijft u dat de handelingen “without surgically inflicted damage” plaatsvinden . Bij punt D refereert u aan “all surgical procedures” . Kunt u bevestigen dat deze handelingen zonder chirurgie plaatsvinden? Mocht er wel chirurgie noodzakelijk zijn dan zou dit consequenties voor de ongerief inschatting “licht” kunnen hebben, kunt u dit toelichten?

Mijn antwoord: “all surgical procedures” onder punt D refereert naar de procedures die nodig zijn om de vector te injecteren en refereert niet naar het maken van een laesie. Het maken van een laesie is in dit type dierproef niet aan de orde. Dit is nu verwoord in de tekst van 3.4.4.4 onder D waar is toegevoegd: ….REQUIRED FOR VECTOR INJECTION….

Uw vraag (11 juni): U beschrijft het gebruik van ratten en muizen, de CCD hecht eraan het aantal in voorraad gedode dieren terug te dringen en heeft daarom de vraag of u in zijn algemeenheid kunt toelichten of u (behalve voor de fok); voor de experimentele procedures beide geslachten kunt gebruiken of in het geval u 1 geslacht gebruikt motiveren waarom dit is?

Mijn antwoord: we streven ernaar transgene lijnen te genereren en in stand te houden met een minimaal fokoverschot en gebruiken hiervoor zowel mannen als vrouwen. De experimentele procedures met *muizen* beschreven in Appendices 1 t/m 4 worden uitgevoerd op zowel vrouwen als mannen. Voor *ratten* geldt dat we deze aanschaffen bij een commerciële fokker. Vrijwel al onze experimentele procedures met ratten doen we met vrouwen omdat we willen uitsluiten dat er extra variatie ontstaat t.g.v. eventuele geslachtsverschillen.

Uw vraag (11 juni): In Bijlage 3.4.4.5. beschrijving dierproeven is punt H. niet volledig ingevuld, wij interpreteren dit als dat er NO in plaats van YES aangekruist had moeten worden.

Mijn antwoord: De muizen in Appendix 5 opgevoerd voor ongeriefmonitoring (n=840) zullen naar onze inschatting geen pijn ondervinden (licht ongerief) . Echter de muizen benodigd voor het genereren van TG lijnen (n=2250; gevasectomeerde mannen, donoren, fosters) zullen wel pijn ondervinden (matig ongerief). Zie ook de bijlage “Bijlage Table of Groups”. Dit is de reden waarom we YES hebben aangekruist. De vraag is hoe binnen de kaders van het formulier duidelijk te maken dat er ook dieren zijn waarvoor NO van toepassing is. Ons voorstel is om zowel NO als YES aan te kruisen met een korte toelichting onder het YES met de volgende tekst: “Animals involved in welfare assessment (n = 840) will experience no pain. Animals involved in the generation of TG lines (n=2250) will experience pain as a consequence of the procedures and they receive adequate anaesthesia and analgesia.”

Hopende u hiermee voldoende te hebben geïnformeerd.

Met vriendelijke groet,

[redacted]

[redacted]

[redacted]

cc. DEC-KNAW

15

Centrale Commissie Dierproeven

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ZBO-CCD@minez.nl

Onze referentie
Aanvraagnummer
AVD801002015104

Uw referentie
-

Bijlagen
1

Datum 24 juni 2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 22 mei 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven digitaal ontvangen. Het gaat om uw project "Developing strategies to promote repair or plasticity of the central and peripheral nervous system" met aanvraagnummer AVD801002015104. Wij hebben uw aanvraag beoordeeld.

Op 12 juni 2015 heeft u uw aanvraag aangevuld op basis van door het secretariaat van de CCD gestelde vragen.

Beslissing
Wij keuren uw aanvraag onder voorwaarde goed op grond van artikel 10a van de Wet op de dierproeven (hierna de wet). U kunt met uw project "Developing strategies to promote repair or plasticity of the central and peripheral nervous system" starten. De vergunning wordt afgegeven van 1 juli 2015 tot en met 30 juni 2020.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-KNAW gevoegd. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze gegevens in het colofon.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend: - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: KNAW, Nederlands Herseninstituut
Adres: Postbus 19121
Postcode en woonplaats: 1000 GC Amsterdam
Deelnemersnummer: 80100

deze projectvergunning voor het tijdvak 1 juli 2015 tot en met 30 juni 2020, voor het project "Developing strategies to promote repair or plasticity of the central and peripheral nervous system" met aanvraagnummer AVD801002015104, volgens advies van dierexperimentencommissie DEC-KNAW.
De functie van de verantwoordelijk onderzoeker is group leader, group regeneration of sensory motor systems.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 22 mei 2015.
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 22 mei 2015;
   b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 22 mei 2015;
   c. Advies van dierexperimentencommissie d.d. 22 mei 2015, ontvangen op 22 mei 2015.
   d. Aanvullingen ontvangen op 12 juni 2015

Dierproeven

Naam dierproef	**Diersoort**	**Aantal dieren**	**Ernst**
Dierproef 1: Molecular screens: Sacrifice of mice or rats following a lesion and/or intervention with a viral vector to obtain neural tissue for molecular screens	Ratten- adult Muizen -adult	240 240	Matig Matig
Dierproef 2: Sacrifice of embryos of rats or mice or of adult rats or mice to obtain tissues for cell culture and bioassays.	Ratten –adult Ratten-embryo Ratten- P1-P7 Muizen –adult Muizen-embryo Muizen- P1-P7	225 1000 375 225 1000 375	Licht Licht Licht Licht Licht Licht
Dierproef 3: Injury models (functional and histological analysis): Injection of cells or viral vectors in lesioned animals.	Ratten- adult Muizen -Adult	900 900	Matig Matig
Dierproef 4: Testing of the quality of viral vector batches	Ratten –Adult Muizen -Adult	460 180	Licht Licht
Dierproef 5: Monitoring and generation of novel genetically modified mice	Muizen- adult *Welfare assessment* Muizen –adult *Genereren transgenen*	840 2250	Licht Matig

Aanvullende voorwaarden:
In Artikel 10, eerste lid, onder a, Wet op de dierproeven, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Weergave wet- en regelgeving

Dit project en wijzigingen
Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt.
Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd.

Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.

16

[redacted]

Van: ZBO-CCD
Verzonden: vrijdag 22 mei 2015 14:32
Aan: 'secretariaat DEC'
CC: [redacted]
Onderwerp: ontvangstbevestiging AVD801002015104
Bijlagen: ontvangsbevestiging aanvraag projectvergunning dierproeven AVD 801002015104.pdf

Geachte heer/mevrouw,

Hierbij zenden wij u per mail een ontvangstbevestiging AVD/801002015104: Ontwikkeling van strategieën om zenuwweefselregeneratie te bevorderen.
Deze zal ook per post worden verzonden.

Met vriendelijke groet,

Centrale Commissie Dierproeven www.zbo-ccd.nl
[redacted]

Postbus 20401 | 2500 EK | Den Haag

17

[redacted]

Van: ZBO-CCD
Verzonden: vrijdag 5 juni 2015 15:40
Aan: [redacted]
Onderwerp: tekstuele aanpassing AVD801002015104

Geachte meneer [redacted]

Zojuist heb ik gevraagd om enkele tekstuele verduidelijking voor projectaanvraag: Developing strategies to promote repair or plasticity of the central and peripheral nervous system" met nummer AVD 801002015104 . Omdat dit om enkel tekstuele verduidelijking gaat en niet om inhoudelijke vraagstelling wordt deze mail parallel aan aanvrager en DEC gestuurd,

Vraag:
In de beschrijving dierproeven 3.4.4.3 ; Injury models: Bij punt B beschrijft u in de eerste alinea een aantal van 750 genetisch gemodificeerde of WT muizen. Verderop in de tekst berekend u voor de injury models 900 ratten en 900 muizen. Het wordt uit de tekst niet helemaal helder of het totale aantal 750 GGO muizen + 900 ratten + 900 muizen is. Kunt u dit toelichten en als nodig in de tekst aanpassen?

In beschrijving dierproeven 3.4.4.4; testing of the quality of viral vector batches, zit een tekstuele inconsequentie: bij punt A beschrijft u dat de handelingen "without surgically inflicted damage" plaatsvinden . Bij punt D refereert u aan "all surgical procedures" . Kunt u bevestigen dat deze handelingen zonder chirurgie plaatsvinden? Mocht er wel chirurgie noodzakelijk zijn dan zou dit consequenties voor de ongerief inschatting "licht" kunnen hebben, kunt u dit toelichten?

Vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.zbo-ccd.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....

[redacted]

Van: ZBO-CCD
Verzonden: donderdag 11 juni 2015 14:47
Aan: [redacted]
Onderwerp: FW: AVD801002015104 tekstuele uitleg/aanpassing

Geachte [redacted]

In aanvulling op onderstaande mail betreffende uw project: Developing strategies to promote repair or plasticity of the central and peripheral nervous system" met nummer AVD 801002015104. Zijn er nog een paar vragen. Excuses dat deze beoordeling gefaseerd gaat, hopelijk is het voor u nog mogelijk de antwoorden in een keer te doen en hebben deze berichten elkaar niet gekruist.

U beschrijft het gebruik van ratten en muizen, de CCD hecht eraan het aantal in voorraad gedode dieren terug te dringen en heeft daarom de vraag of u in zijn algemeenheid kunt toelichten of u (behalve voor de fok) ; voor de experimentele procedures beide geslachten kunt gebruiken of in het geval u 1 geslacht gebruikt motiveren waarom dit is?

In Bijlage 3.4.4.5. beschrijving dierproeven is punt H. niet volledig ingevuld, wij interpreteren dit als dat er NO in plaats van YES aangekruist had moeten worden.

Met vriendelijke groet [redacted]

Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....

Van: ZBO-CCD
Verzonden: vrijdag 5 juni 2015 15:33
Aan: [redacted]
Onderwerp: AVD801002015104 tekstuele uitleg/aanpassing

Geachte [redacted]

Uw aanvraag getiteld ; Developing strategies to promote repair or plasticity of the central and peripheral nervous system" met nummer AVD 801002015104 is ontvangen en in behandeling genomen. Er zijn twee tekstuele onduidelijkheden in de bijlage dierproeven waarvan we u willen vragen deze te verduidelijken en aan te passen zodat het aantal dieren en ongerief inschatting met elkaar in overeenstemming is.

In de beschrijving dierproeven 3.4.4.3 ; Injury models: Bij punt B beschrijft u in de eerste alinea een aantal van 750 genetisch gemodificeerde of WT muizen. Verderop in de tekst berekend u voor de injury models 900 ratten en 900 muizen. Het wordt uit de tekst niet helemaal helder of het totale aantal 750 GGO muizen + 900 ratten + 900 muizen is. Kunt u dit toelichten en als nodig in de tekst aanpassen?

In beschrijving dierproeven 3.4.4.4; testing of the quality of viral vector batches, zit een tekstuele inconsequentie: bij punt A beschrijft u dat de handelingen "without surgically inflicted damage" plaatsvinden . Bij punt D refereert u aan "all surgical procedures" . Kunt u bevestigen dat deze handelingen zonder chirurgie plaatsvinden? Mocht er wel chirurgie noodzakelijk zijn dan zou dit consequenties voor de ongerief inschatting "licht" kunnen hebben, kunt u dit toelichten?

Met vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.zbo-ccd.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....

[redacted]

Van: secretariaat DEC [redacted]
Verzonden: vrijdag 12 juni 2015 13:28
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD-801002015-104 tekstuele uitleg/aanpassing

Categorieën: [redacted]

Geachte mevrouw [redacted]

Namens [redacted] heb ik zojuist via Webftp een aantal files gestuurd met een brief met de antwoorden op de gestelde vragen alsmede een bijstelling van een aantal bijlagen en een tabel met een overzicht van het aantal dieren.

Met vriendelijke groeten,

[redacted] DEC-KNAW

From: [redacted]
Sent: Thursday, June 11, 2015 2:49 PM
To: secretariaat DEC
Subject: FW: AVD801002015104 tekstuele uitleg/aanpassing

Geachte heer [redacted]

Onderstaande mail is zojuist aan de onderzoeker verzonden met het verzoek op het punt van het gebruik van beide geslachten nog een toelichting te geven.

Vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....

Van: ZBO-CCD
Verzonden: donderdag 11 juni 2015 14:47
Aan: [redacted]
Onderwerp: FW: AVD801002015104 tekstuele uitleg/aanpassing

Geachte [redacted],

In aanvulling op onderstaande mail betreffende uw project: Developing strategies to promote repair or plasticity of the central and peripheral nervous system" met nummer AVD 801002015104. Zijn er nog een paar vragen. Excuses dat deze beoordeling gefaseerd gaat, hopelijk is het voor u nog mogelijk de antwoorden in een keer te doen en hebben deze berichten elkaar niet gekruist.

U beschrijft het gebruik van ratten en muizen, de CCD hecht eraan het aantal in voorraad gedode dieren terug te dringen en heeft daarom de vraag of u in zijn algemeenheid kunt toelichten of u (behalve voor de fok) ; voor de

experimentele procedures beide geslachten kunt gebruiken of in het geval u 1 geslacht gebruikt motiveren waarom dit is?

In Bijlage 3.4.4.5. beschrijving dierproeven is punt H. niet volledig ingevuld, wij interpreteren dit als dat er NO in plaats van YES aangekruist had moeten worden.

Met vriendelijke groet [redacted]

Centrale Commissie Dierproeven
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.....
Postbus 20401 | 2500 EK | Den Haag
.....

Van: ZBO-CCD
Verzonden: vrijdag 5 juni 2015 15:33
Aan: [redacted]
Onderwerp: AVD801002015104 tekstuele uitleg/aanpassing

Geachte [redacted]

Uw aanvraag getiteld ; Developing strategies to promote repair or plasticity of the central and peripheral nervous system" met nummer AVD 801002015104 is ontvangen en in behandeling genomen. Er zijn twee tekstuele onduidelijkheden in de bijlage dierproeven waarvan we u willen vragen deze te verduidelijken en aan te passen zodat het aantal dieren en ongerief inschatting met elkaar in overeenstemming is.

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In beschrijving dierproeven 3.4.4.4; testing of the quality of viral vector batches, zit een tekstuele inconsequentie: bij punt A beschrijft u dat de handelingen "without surgically inflicted damage" plaatsvinden . Bij punt D refereert u aan "all surgical procedures" . Kunt u bevestigen dat deze handelingen zonder chirurgie plaatsvinden? Mocht er wel chirurgie noodzakelijk zijn dan zou dit consequenties voor de ongerief inschatting "licht" kunnen hebben, kunt u dit toelichten?

Met vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.zbo-ccd.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....

20

[redacted]

Van: ZBO-CCD
Verzonden: donderdag 18 juni 2015 16:37
Aan: [redacted]
Onderwerp: betaling AVD2015104 mogelijk dubbel ontvangen

Geachte [redacted]

Voor uw project aanvraag hebben wij de leges ontvangen, maar uit onze administratie blijkt dat wij deze betaling mogelijk 2x hebben ontvangen op zowel 9-6-15 als op 16-6-15. Omdat ik geen gegevens van uw financiële afdeling heb hoop ik dat u deze vraag door kunt sturen.
Ik voeg de betalingskenmerken bij,

[redacted]

[redacted]

Vriendelijke groet, [redacted]

Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....

	Inventaris Wob-verzoek W16-01								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2015105								
1	Aanvraagformulier				x		x	x	
2	Niet-technische samenvatting	x							
3	Aanvraag aanvullende informatie				x		x	x	
4	Bijlage dierproeven 1				x		x	x	
5	Bijlage dierproeven 2				x		x	x	
6	Bijlage dierproeven 3				x		x	x	
7	Bijlage dierproeven 4				x		x	x	
8	Roadmap/ schematishce weergave project				x		x	x	
9	Tabel ongerief classificatie dierproef 1t/m 4			x					
10	DEC-advies				x		x	x	
11	Mailwisseling 1 17-6-2015				x		x	x	
12	Mailwisseling 2 12-6-2015				x		x	x	
13	Mailwisseling 3 16-6-2015				x		x	x	
14	Mailwisseling 4 17-6-2015				x		x	x	
15	Mailwisseling 5 16-7-2015				x		x	x	
16	Aanvullende reactie				x		x	x	
17	Advies CCD		x						x
18	Beschikking				x		x	x	
19	Vergunning			x					
20	Ontvangstbevestiging				x		x	x	
21	Rappelbrief aanvullende informatie				x		x	x	

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om een of meerdere dierproeven uit te voeren
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren Of u geeft aan wat u in het vergunde project wilt wijzigen
- Meer informatie over de voorwaarden vindt u op de website www zbo ccd nl of in de toelichting op de website
- Of bel met 0900 2800028 (10 ct/min)

1 Gegevens aanvrager

1 1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA

☑ Ja > *Vul uw deelnemernummer in* 80101 (Nederlands Hersenınstituut KNAW)
☐ Nee > *U kunt geen aanvraag doen*

1 2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt

Naam instelling of organisatie	KNAW
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK nummer	5 4 6 6 7 0 8 9

1 3 Vul de gegevens van het postadres in
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker

Straat en huisnummer	
Postbus	Postbus 19121
Postcode en plaats	1000GC Amsterdam
IBAN	NL33DEUT0546900054
Tenaamstelling van het rekeningnummer	Nederlands Hersenınstituut

1 4 Vul de gegevens in van de verantwoordelijke onderzoeker

(Titel) Naam en voorletters	☑ Dhr ☐ Mw [redacted]
Functie	Group Leader
Afdeling	[redacted]
Telefoonnummer	[redacted]
E mailadres	[redacted]

1 5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker

(Titel) Naam en voorletters	☐ Dhr ☑ Mw [redacted]
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E mailadres	[redacted]

1 6	*(Optioneel)* Vul h er de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning	(Titel) Naam en voorletters Functie Afdeling Telefoonnummer E mailadres	☐ Dhr ☑ Mw
1 7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ngevulde formulier Meld ng Macht g ng mee met deze aanvraag* ☑ Nee	

2 Over uw aanvraag

2 1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3 ☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2 2 ☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2 3
2 2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier ☑ Nee > Ga verder met vraag 3
2 3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☑ Nee > Ga verder met vraag 3 ☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3 1	Wat is de geplande start en einddatum van het project?	Startdatum 0 1 - 0 / 2 0 1 5 Einddatum 0 1 0 / 2 0 2 0
3 2	Wat is de titel van het project?	Multilevel investigation of the neural correlates of emotion understanding in rodents
3 3	Wat is de titel van de niet technische samenvatting?	Neuronale basis van emotie
3 4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC DEC KNAW Postadres [redacted] Amsterdam E mailadres [redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- [x] Nieuwe aanvraag Projectvergunning € 741,00 Lege
- [ ] Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

- [x] Via een eenmalige incasso
- [ ] Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- [x] Projectvoorstel
- [x] Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- [ ] Melding Machtiging
- [ ] Appendix 4 maal en Roadmap

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted]
Plaats	Amsterdam
Datum	0 1 - 0 6 - 2 0 1 5
Handtekening	[redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
t.a.v. [redacted]
Postbus 19121
1000GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015105

Datum 17 juni 2015
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Bijlagen
1

Geachte [redacted]

Op 5 juni 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project “Multilevel investigation of the neural correlates of emotion undestanding in rodents” met aanvraagnummer AVD801002015105. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

Welke informatie nog nodig
Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen:

Onduidelijkheden

Dierproef 3.4.4.1

1) In uw aanvraag schrijft u van plan te zijn om verschillende stammen en lijnen ratten en muizen te testen. Zou u kunnen toelichten waarom is het noodzakelijk om meerdere stammen/lijnen ratten en muizen te gebruiken om een test te valideren en in hoeverre de verschillen tussen de stammen/lijnen invloed kunnen hebben op de te valideren testen?

2) Als onderdeel van uw dierproef bent u van plan om dieren met bepaalde karakteristieken te fokken. We zullen graag meer informatie van u willen ontvangen over het doel van deze proef, de karakteristieken die gekozen worden en de manier waarop dat gebeurt. Kan deze proef leiden tot een nieuwe stam en/ of kunnen de nakomelingen ongerief hebben? Wat gebeurt er met de nakomelingen?

3) Onder het aantal dieren nodig voor de gedragstesten schrijft u dat er 1600 ratten en 1600 muizen nodig zijn om 3 positieve en 3 negatieve emoties te testen

en ook voor het testen van de instrumentele agressie. Voor elk gedrag meldt u 200 dieren nodig te hebben. Zou u kunnen uitleggen hoeveel groepen bent u van plan te gebruiken? Daarnaast schrijft u dat de meerderheid van de dieren in maximaal drie proeven zal worden gebruikt. Bedoelt u hier dat elk dier in maximaal drie gedragstesten per emotie wordt getest of in maximaal drie verschillende positieve/negatieve emoties?

Alle dierproeven:

4) In de beschrijving van de dierproeven schrijft u dat een groot deel van de behandelingen optioneel zijn. Zou u kunnen uitleggen op basis waarvan u al dan niet kiest voor die behandelingen?

5) U bent van plan om mannelijke en vrouwelijke dieren te gebruiken? Geldt dit voor alle experimenten?

6) In hoeverre zou het mogelijk zijn om enkele dierproeven te combineren, om in dezelfde dieren de neuronale activiteit op basisniveau te onderzoeken alsook te manipuleren? Zou dat niet betrouwbaardere resultaten en minder variatie kunnen opleveren, alsook leiden tot minder gebruikte dieren?

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Stuurt u het per post op, gebruik dan het formulier dat u bij deze brief krijgt.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:
- formulier Melding Bijlagen via de post

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

- U wilt één of meerdere bijlagen naar ons versturen? Voeg *altijd* deze Melding Bijlagen toe. Wij weten dan welke documenten van u zijn en hoeveel documenten u opstuurt.
- Meer informatie vindt u op www.centralecommissiedierproeven.nl
- Of bel met ons: 0900 28 000 28 (10 ct/min).

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

1.2 Bij welke aanvraag hoort de bijlage?
Het aanvraagnummer staat in de brief of de ontvangstbevestiging.

Aanvraagnummer	

2 Bijlagen

2.1 Welke bijlagen stuurt u mee?
Vul de naam of omschrijving van de bijlage in.

☐	
☐	
☐	

3 Ondertekening

3.1 Onderteken het formulier en stuur het met alle bijlagen op naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix ‘description animal procedures’ should be enclosed for each type of animal procedure.
- For more information, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the ‘Netherlands Food and Consumer Product Safety Authority’.	80101
1.2	Provide the name of the licenced establishment.	KNAW
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 Type of animal procedure: Measurement of social, prosocial and antisocial behavior

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

-The first step will always be to develop and characterize a behavioral paradigm for measuring behavioral correlates of emotional contagion for positive or negative emotions, prosocial behavior or antisocial behavior. Because the behaviors when fully characterized will be combined with measurement of brain activity, each of the paradigms needs to be adapted and in some cases modified for this

purpose. [redacted]

-Most of these experiments involve the use of pairs of animals in order test how the emotional/empathic behavior of one of them relates to the emotional/empathic behavior of the other. [redacted] Overall, we will measure changes of a particular behavior of interest and the relationship between the changes in the behavior in one animal to changes in the behavior of the other. In addition, we will characterize these responses [redacted] and investigate whether these parameters change as a consequence of the paradigm. In some experimental animals we will also [redacted] To correlate the responses in the observer animals with those in the demonstrators in some cases (<10%) the [redacted] are also performed in the demonstrators.

-Given that both rats and mice are used in the literature to investigate emotional contagion, they will be both used for all types of experiments. [redacted] and thus, to draw a conclusion, it is critical [redacted]

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Animals (e.g., observer-demonstrator) are housed together for a period up to 15 weeks. They are handled every week. If animals undergo a surgical procedure, their weights will be recorded at least once a week, to monitor recovery.

2. (optional) [redacted] (3.4.4.2, 3.4.4.4). Also, if we need [redacted]

3. (optional) Animals are equipped [redacted]

4. (optional) Females will be used in some cases, first to compare behaviors between sexes and second to [redacted] (mainly in mice). In this case females (under proper anaesthesia and perioperative analgesia) [redacted]

5. [redacted]

6. For prosocial and antisocial behavioral paradigms, [redacted]

7. For emotional contagion tests: [redacted]

8. For prosocial tests: [redacted]

9. For antisocial tests: [redacted]

10. (optional) [redacted]

11. (optional) [redacted]

12. (optional) [redacted]

For all our experiments the majority of animals (95%) will undergo a maximum of 3 tests.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

We strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with a t test with a $p<0.05$.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

The rat and mice (males/females) are the best investigated mammal species used for fundamental research, the latest technologies for investigating brain mechanisms are made for use in these species and a variety of KO and transgenic models are available to study brain development, and disease etc. Moreover, there is significant knowledge and extensive characterization of the anatomy and [redacted]

Overall it is important to take into account that in the majority of our experiments we use pairs of animals, [redacted] This means that in most cases twice the number of animals is needed.
There are multiple reasons for using rats and/or mice for the different experiments. First, results from our lab show significant differences in the emotional behavior of mice and rats, we believe this is due to differences in social structures. Investigating these differences will give us a better insight into the neural correlates of these behaviours and how this relates to social structures and environments (isolated and territorial in mice vs grouped/colonies in rats). For this reason we would like to use both rats and mice.
In addition, there is a [redacted]. However, in the near future we aim at [redacted]. On the other hand, [redacted]

Development of behavioral paradigms and characterization of the behavior in **rats** (young adults, adults, [redacted]. We have used rats in the past and they are a good animal model for investigating empathy. Estimated number is 1600 rats. Based on our experience on developing and characterizing the behavioral paradigm for [redacted] Thus, for development and measuring each behavior of interest we estimate we will need ca. 200 animals. [redacted]

- Development of behavioral paradigms and characterization of the behavior in **mice** [redacted] Given that the majority of genetic technologies are much more developed in mice, we also want to do behavioral characterizations in mice. Similar to rats we estimate we will use 1600 mice, [redacted]

- [redacted]

[redacted]

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The original experiments which paved the way for developing this line of research on mirror neurons for actions were performed on monkeys, in this research project we intend on using 'lower' mammals like rats and mice. Choice for rats and mice is mainly practical (small, tools and GMO animals available). Given the complex nature of emotions and feelings it would be impossible to test this in an invertebrate animal. Alternatively, testing this in a computer model is not an option for the moment, given that little to nothing is known regarding the neural mechanisms of empathy, prosocial and antisocial behaviors. We have tested the possibility of using audio/video recordings to replace the demonstrators, but the observer animals do not respond, making it not possible to apply this option. However, in the future one of the aims is to develop computer models that would simulate the neural responses, which would help reducing the number of animals used. [redacted] Each one of the experiments is carefully designed, evaluated by a team of experts and well-controlled to avoid having to repeat the study and use more animals than strictly necessary. Currently, there are no (replacement) alternatives to these experimental methodologies. However, we believe that the knowledge gained from this project will serve as a foundation for therapeutic advances in a variety of psychiatric disorders where alterations in empathy are one of the main problems. Despite the invasive nature of the experiments, we take all the possible measures so that the discomfort of the animals will be as limited as possible. Analgesic and prophylaxis therapies will be administered in the post-surgical stage. Care will be taken to ensure that housing and experimental conditions will be as comfortable as possible to the animals.
All experiments are designed in such a way that multiple pilots are conducted (in a go / no go way) before the full test, to make sure that each experimental parameter is understood and accounted, variability is minimal and that scenarios where data of animals is lost due to unexpected circumstances is extremely rare. This is critical for both refining the experiment and reducing the total number of animals used.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

- All animals from this experiment will be handled every other day starting 10 days prior to experiment start and habituated to the experimental room and setup to reduce animals stress.
- For animals experiencing surgery, analgesic and prophylaxis therapies will be administered during and in the post-surgical stage. Also after longer surgeries animals will be rehydrated by administration of saline subcutaneously, they will be kept in a warm blanket until they wake up and then given soft pellet food (soaked in water) on the floor of the home cage to facilitate easy access to food.
- Animals will be allowed to recover for at least 1 week following the surgery.
- Animal's behaviour, wound area and physiological parameters will be monitored daily for at least 3 days post-surgery and weekly for 3 weeks after surgery.
- [redacted]
- Objects for enrichment will be placed in their home cage.

The welfare of the animals will be constantly monitored and kept as a priority.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

[redacted]. Also, animals will be housed in solitary for a maximum of 3 days until they fully recuperate and then they will be placed back with their partner.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

In some experiments mild [redacted]

In all other cases (surgical interventions) adequate analgesia will be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anaesthesia and analgesia is used for all procedures that are not related to experimental testing, which is primarily surgery.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Insufficient recovery after surgery: It will be considered if animal shows permanent weight loss (more than 15% of the weight immediately after surgery for more than 10 days). –Infrequent occurrence (<2% of animals)
2. Infection: Although we will perform the surgeries in semi-sterile conditions, there is a possibility of infection around the wound area. – Infrequent occurrence (<2%). Visible signs of pathogenesis will be monitored. The following will be considered as signs of unhealthy state of the animal:
   a. Aberrant behaviour
   b. Shock
   c. Dehydration
   d. Weight loss
   e. Nose and mouth discharge

Explain why these effects may emerge.

Result from surgical interventions

Indicate which measures will be adopted to prevent occurrence or minimise severity.

- Constant monitoring of animal's behavior, wound area and physiology together with adequate surgical procedures semi-sterile conditions, fast to minimize amount of time animal is under anesthesia).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Each animal that undergoes surgery will be monitored and scored for clinical parameters.
The clinical parameters are:
Appearance
0 = normal; 1 = unkept fur; 2 = stooped posture; 3 = hair standing up; 4= sunked flanks, visible ribs
Activity level
0 = normal; 1 = small reduction in activity; 2 = large reduction in activity; 3 = immobility
Recovery
0 = recovered from anesthesia; 1 = not recovered from anesthesia
Wound
0 = normal; 1 = inflammation; 2 = red and infected

An animal with a score of 3 or higher in any of the categories or a combined score of 5 will closely monitored and evaluated together with the veterinarian to decide if a humane endpoint should be conducted

In addition weight will be monitored and if the animal loses weight of 10-15% in 2 consecutive days or if the animal loses more than 20% of weight throughout the course of the experiment then the veterinarian of the NIN will be contacted and a decision will be made.

[redacted]

Indicate the likely incidence.

[redacted]

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

[redacted]

[redacted]

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be sacrificed after the procedures to collect brains for post-mortem investigation of brain activity.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- ☐ This appendix should be enclosed with the project proposal for animal procedures.
- ☐ A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- ☐ For more information, see our website (www.zbo-ccd.nl).
- ☐ Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101
1.2	Provide the name of the licenced establishment.	KNAW
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 Type of animal procedure: Measure activity at the neuronal, brain region or network level in different behavioral paradigms (behavior + neuronal activity measurement)

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

While participating in an empathy behaviour task (see 3.4.4.1) [redacted]

[redacted] These measurements will provide rich information that would allow us to understand the neural correlates of the behaviors in question. These measurements will only take place in combination with a behaviour task that has proven to show the desired behaviour.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. In this procedure all animals will participate in a behavioural task as described in 3.4.4.1
2. [redacted]
3. (optional) [redacted]
4. (optional) [redacted]
5. (optional) [redacted]
6. [redacted]
7. [redacted]
8. (optional) [redacted])
9. [redacted]

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

To estimate the number of animals to be used in an experiment, we use the effect size (if known e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with a certain degree confidence interval with a t test with a $p<0.05$. In cases where the effect size is unknown, then the effect size is obtained from published studies that use similar behavioral paradigms.

Most experiments use pairs of animals as a unit, however, brain activity measurements are mostly performed in the observer animals, however in each case also demonstrator animals are required. This means that in most cases twice the number of animals as calculated from the power analyses is needed.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Overall it is important to take into account that in the majority of our experiments we use pair of animals, which represents a significant increase in the total number of animals used.

- Rats from commercial companies ([redacted]. The rats used will be young adults, adults. We have successfully used [redacted], we believe with the other behavioral paradigms and techniques we will have the same success with these specie. We estimate we will need a total of 1200 animals. [redacted]

[redacted]. The mice used will be young adults and adults. In contrast, to rats, a variety of genetic tool are readily available for use in mice. [redacted]

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The original experiments which paved the way for developing this line of research on mirror neurons for actions were performed on monkeys, in this research project we intend on

using ‘lower’ mammals like rats and mice. Choice for rats and mice is mainly practical (small, tools and GMO animals available). Given the complex nature of emotions and feelings it would be impossible to test this in an invertebrate animal. We have tested the possibility of using audio/video recordings to replace the demonstrators, but the observer animals do not respond, making it not possible to apply this option. Alternatively, testing this in a computer model is not an option for the moment, given that little to nothing is known regarding the neural mechanisms of empathy, prosocial and antisocial behaviors. However, in the future one of the aims is to develop computer models that would simulate the neural responses, which would help reducing the number of animals used.
Currently, the most studied and well [redacted] Each one of the experiments is carefully designed, evaluated by a team of experts and well-controlled to avoid having to repeat the study and use more animals than strictly necessary.
There are no (replacement) alternatives to these experimental methodologies. However, we believe that the knowledge gained from this project will serve as a foundation for therapeutic advances in a variety of psychiatric disorders where alterations in empathy are one of the main problems. Despite the invasive nature of the experiments, we take all the possible measures so that the discomfort of the animals will be as limited as possible. Analgesic and prophylaxis therapies will be administered in the post-surgical stage. Care will be taken to ensure that housing and experimental conditions will be as comfortable as possible to the animals.
All experiments are designed in such a way that multiple pilots are conducted (in a go no go way) before the full test, to make sure that each experimental parameter is understood and accounted, variability is minimal and that scenarios where data of animals is lost due to unexpected circumstances is extremely rare . This is critical for both refining the experiment and reducing the total number of animals used.
The main reduction in the number of animals used is achieved by using [redacted] thereby further reducing the number of animals used.
There are no alternatives to these invasive experimental methodologies capable of providing the same kind of information about the neural code for emotion processing. However, we believe that the knowledge gained from this project will serve as a foundation for therapeutic advances in a variety of psychiatric disorders where alterations in empathy are one of the main problems. Despite the invasive nature of the experiments, we take all the possible measures so that the discomfort to the animals will be as limited as possible. Analgesic and prophylaxis therapies will be administered in the post-surgical stage. Care will be taken to ensure that housing and experimental conditions will be as comfortable as possible to the animals.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

- All animals from this experiment will be handled every other day starting 10 days prior to experiment start and habituated to the experimental room and setup to reduce animals stress.
- For animals experiencing surgery, analgesic and prophylaxis therapies will be administered during and in the post-surgical stage. Also after longer surgeries animals will be rehydrated by administration of saline subcutaneously, they will be kept in a warm blanket until they wake up and then given soft pellet food (soaked in water) on the floor of the home cage to facilitate easy access to food.

-Animals will be allowed to recover for at least 1 week following the surgery.
-Animal’s behaviour, wound area and physiological parameters will be monitored daily for at least 3 days post-surgery and weekly for 3 weeks after surgery.
[redacted]

- Objects for enrichment will be placed in their homecage.
The welfare of the animals will be constantly monitored and kept as a priority

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

[redacted]

Also, [redacted], animals will be housed in solitary for a maximum of 3 days until they fully recuperate and then they will be placed back with their partner.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

[redacted]

In all other cases (surgical interventions) adequate analgesia will be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anaesthesia and analgesia is used for all procedures that are not related to experimental testing, which is primarily surgery .

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Insufficient recovery after surgery: It will be considered if animal shows permanent weight loss (more than 15% of the weight immediately after surgery for more than 10 days). –Infrequent occurrence (<2% of animals)
2. Infection: Although we will perform the surgeries in semi-sterile conditions, there is a possibility of infection around the wound area. –Infrequent occurrence (<2%). Visible signs of pathogenesis will be monitored. The following will be considered as signs of unhealthy state of the animal:
   a. Aberrant behaviour
   b. Shock
   c. Dehydration
   d. Weight loss
   e. Nose and mouth discharge

Explain why these effects may emerge.

Result from surgical interventions

Indicate which measures will be adopted to prevent occurrence or minimise severity.

- Constant monitoring of animal's behavior, wound area and physiology together with adequate surgical procedures semi-sterile conditions, fast to minimize amount of time animal is under anesthesia).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Each animal that undergoes surgery will be monitored and scored for clinical parameters.
The clinical parameters are:
Appearance
0 = normal; 1 = unkept fur; 2 = stooped posture; 3 = hair standing up; 4= sunked flanks, visible ribs
Activity level
0 = normal; 1 = small reduction in activity; 2 = large reduction in activity; 3 = immobility
Recovery
0 = recovered from anesthesia; 1 = not recovered from anesthesia

Wound
0 = normal; 1 = inflammation; 2 = red and infected

An animal with a score of 3 or higher in any of the categories or a combined score of 5 will closely monitored and evaluated together with the veterinarian to decide if a humane endpoint should be conducted

In addition weight will be monitored and if the animal loses weight of 10-15% in 2 consecutive days or if the animal loses more than 20% of weight throughout the course of the experiment then [redacted] will be contacted and a decision will be made.
Animals that do not adapt [redacted] will be removed from the experiment a[redacted]

Indicate the likely incidence.

Animals with ovariectomy (<2%)
Animals with surgical implantation of telemetry device <10%
Animals with headpost (<2%)
Animals that do not adapt to head-restrain (<5%)

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

[redacted]

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: 'signatures'.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be sacrificed after the procedures to collect brains for post-mortem investigation of brain activity.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
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- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the ‘Netherlands Food and Consumer Product Safety Authority’.	80101
1.2	Provide the name of the licenced establishment.	KNAW
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.3 Type of animal procedure: Manipulate/alter and then measure the behavioral consequences in different behavioral paradigms

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

[redacted]

[redacted]

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Pre-experimental + testing manipulations

   a. [redacted]

   b. [redacted]

   c. [redacted]

2. All animals will participate in behavioural tasks as described in 3.4.4.1

[redacted]. [redacted]

(optional) [redacted]

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

We strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with a t test with a $p<0.05$.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Overall it is important to take into account that in the majority of our experiments we use pair of animals, which represents a significant increase in the total number of animals used.

We will use rats that are young adults, adults and dams [redacted] For these manipulation experiments we estimate that we will use approximately 1200 animals. In particular, the [redacted] will require a larger number of animals (40-60% of the total) [redacted]

We will also use mice that are young adults, adults and dams (mother-pups for maternal behavior studies) [redacted] Similar to rats, we estimate we will estimate that we will need a total of 1200 animals, [redacted]

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The original experiments which paved the way for developing this line of research on mirror neurons for actions were performed on monkeys, in this research project we intend on using lower mammals. Given the complex nature of emotions and feelings it would be impossible to test this in an invertebrate animal. Alternatively, testing this in a computer model is not an option for the moment, given that little to nothing is known regarding the neural mechanisms of empathy, prosocial and antisocial behaviors. We have tested the possibility of using audio/video recordings to replace the demonstrators, but the observer animals do not respond, making it not possible to apply this option. However, in the future one of the aims is to develop computer models that would simulate the neural responses, which would help reducing the number of animals used.

[redacted]. Each one of the experiments is carefully designed, evaluated by a team of experts and well-controlled to avoid having to repeat the study and use more animals than strictly necessary.

Currently, there are no (replacement) alternatives to these experimental methodologies. However, we believe that the knowledge gained from this project will serve as a foundation for therapeutic advances in a variety of psychiatric disorders where alterations in empathy are one of the main problems. Despite the invasive nature of the experiments, we take all the possible measures so that the discomfort of the animals will be as limited as possible. Analgesic and prophylaxis therapies will be administered in the post-surgical stage. Care will be taken to ensure that housing and experimental conditions will be as comfortable as possible to the animals.

All experiments are designed in such a way that multiple pilots are conducted (in a go no go way) before the full test, to make sure that each experimental parameter is understood and accounted, variability is minimal and that scenarios where data of animals is lost due to unexpected circumstances is extremely rare. This is critical for both refining the experiment and reducing the total number of animals used.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

- All animals from this experiment will be handled every other day starting 10 days prior to experiment start and habituated to the experimental room and setup to reduce animals stress.
- For animals experiencing surgery, analgesic and prophylaxis therapies will be administered during and in the post-surgical stage. Also after longer surgeries animals will be rehydrated by administration of saline subcutaneously, they will be kept in a warm blanket until they wake up and then given soft pellet food (soaked in water) on the floor of the home cage to facilitate easy access to food.

-Animals will be allowed to recover for at least 1 week following the surgery.

-Animal's behaviour, wound area and physiological parameters will be monitored daily for at least 3 days post-surgery and weekly for 3 weeks after surgery.

- [redacted]

- Objects for enrichment will be placed in their home cage.

The welfare of the animals will be constantly monitored and kept as a priority

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

[redacted]

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

[redacted]

In all other cases (surgical interventions) adequate analgesia will be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anaesthesia and analgesia is used for all procedures that are not related to experimental testing, which is primarily surgery.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Insufficient recovery after surgery: It will be considered if animal shows permanent weight loss (more than 15% of the weight immediately after surgery for more than 10 days). –Infrequent occurrence (<2% of animals)
2. Infection: Although we will perform the surgeries in semi-sterile conditions, there is a possibility of infection around the wound area. – Infrequent occurrence (<2%). Visible signs of pathogenesis will be monitored. The following will be considered as signs of unhealthy state of the animal:
   a. Aberrant behaviour
   b. Shock
   c. Dehydration

d. Weight loss
e. Nose and mouth discharge

Explain why these effects may emerge.

Result from surgical interventions

Indicate which measures will be adopted to prevent occurrence or minimise severity.

- Constant monitoring of animal's behavior, wound area and physiology together with adequate surgical procedures semi-sterile conditions, fast to minimize amount of time animal is under anesthesia).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Each animal that undergoes surgery will be monitored and scored for clinical parameters.
The clinical parameters are:
Appearance
0 = normal; 1 = unkept fur; 2 = stooped posture; 3 = hair standing up; 4= sunked flanks, visible ribs
Activity level
0 = normal; 1 = small reduction in activity; 2 = large reduction in activity; 3 = immobility
Recovery
0 = recovered from anesthesia; 1 = not recovered from anesthesia
Wound
0 = normal; 1 = inflammation; 2 = red and infected

An animal with a score of 3 or higher in any of the categories or a combined score of 5 will closely monitored and evaluated together with the veterinarian to decide if a humane endpoint should be conducted

In addition weight will be monitored and if the animal loses weight of 10-15% in 2 consecutive days or if the animal loses more than 20% of weight throughout the course of the experiment then [redacted]

Indicate the likely incidence.

[redacted]

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (‘non-recovery’, ‘mild’, ‘moderate’, ‘severe’).

[redacted]

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: ‘signatures’.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be sacrificed after the procedures to collect brains for post-mortem investigation of brain activity.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix ‘description animal procedures’ should be enclosed for each type of animal procedure.
- For more information, see our website (www.zbo-ccd.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the ‘Netherlands Food and Consumer Product Safety Authority’.	80101
1.2	Provide the name of the licenced establishment.	KNAW
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.4 Type of animal procedure: Manipulate/alter and then measure the consequences in terms of activity at the neuronal, brain region or network level in these animals in different behavioral paradigms

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

[redacted]

[redacted]

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. All animals will participate in the same procedures as described in 3.4.4.3 including:

[redacted]

- (optional) [redacted]

-(optional) [redacted]

- (optional) [redacted]

[redacted]

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

We strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample

size needed to achieve a certain power (usually around 0.8) with a t test with a $p<0.05$.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Overall it is important to take into account that in the majority of our experiments we use pair of animals, which represents a significant increase in the total number of animals used.

- We will use rats that are young adults, adults and dams (mother-pups for maternal behavior studies) f[redacted] For these manipulation experiments we estimate that we will use approximately 300 animals. Most of the rats will be used in combination [redacted]

We will also use mice that are young adults, adults and dams (mother-pups for maternal behavior studies) [redacted]

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

The original experiments which paved the way for developing this line of research on mirror neurons for actions were performed on monkeys, in this research project we intend on using lower mammals. Given the complex nature of emotions and feelings it would be impossible to test this in an invertebrate animal. Alternatively, testing this in a computer model is not an option for the moment, given that little to nothing is known regarding the neural mechanisms of empathy, prosocial and antisocial behaviors. We have tested the possibility of using audio/video recordings to replace the demonstrators, but the observer animals do not respond, making it not possible to apply this option. However, in the future one of the aims is to develop computer models that would simulate the neural responses, which would help reducing the number of animals used.
Currently, the most studied and well [redacted]

[redacted] Each one of the experiments is carefully designed, evaluated by a team of experts and well-controlled to avoid having to repeat the study and use more animals than strictly necessary. Currently, there are no (replacement) alternatives to these experimental methodologies. However, we believe that the knowledge gained from this project will serve as a foundation for therapeutic advances in a variety of psychiatric disorders where alterations in empathy are one of the main problems. Despite the invasive nature of the experiments, we take all the possible measures so that the discomfort of the animals will be as limited as possible. Analgesic and prophylaxis therapies will be administered in the post-surgical stage. Care will be taken to ensure that housing and experimental conditions will be as comfortable as possible to the animals.
All experiments are designed in such a way that multiple pilots are conducted (in a go no go way) before the full test, to make sure that each experimental parameter is understood and accounted, variability is minimal and that scenarios where data of animals is lost due to unexpected circumstances is extremely rare . This is critical for both refining the experiment and reducing the total number of animals used.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

- All animals from this experiment will be handled every other day starting 10 days prior to experiment start and habituated to the experimental room and setup to reduce animals stress.
- For animals experiencing surgery, analgesic and prophylaxis therapies will be administered during and in the post-surgical stage. Also after longer surgeries animals will be rehydrated by administration of saline subcutaneously, they will be kept in a warm blanket until they wake up and then given soft pellet food (soaked in water) on the floor of the home cage to facilitate easy access to food.

-Animals will be allowed to recover for at least 1 week following the surgery.

-Animal's behaviour, wound area and physiological parameters will be monitored daily for at least 3 days post-surgery and weekly for 3 weeks after surgery.

[redacted]

- Objects for enrichment will be placed in their homecage.

The welfare of the animals will be constantly monitored and kept as a priority

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

[redacted]

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

[redacted]

In all other cases (surgical interventions) adequate analgesia will be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anaesthesia and analgesia is used for all procedures that are not related to experimental testing, which is primarily surgery.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Insufficient recovery after surgery: It will be considered if animal shows permanent weight loss (more than 15% of the weight immediately after surgery for more than 10 days). –Infrequent occurrence (<2% of animals)
2. Infection: Although we will perform the surgeries in semi-sterile conditions, there is a possibility of infection around the wound area. – Infrequent occurrence (<2%). Visible signs of pathogenesis will be monitored. The following will be considered as signs of unhealthy

state of the animal:
a. Aberrant behaviour
b. Shock
c. Dehydration
d. Weight loss
e. Nose and mouth discharge

Explain why these effects may emerge.

Result from surgical interventions

Indicate which measures will be adopted to prevent occurrence or minimise severity.

- Constant monitoring of animal's behavior, wound area and physiology together with adequate surgical procedures semi-sterile conditions, fast to minimize amount of time animal is under anesthesia).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Each animal that undergoes surgery will be monitored and scored for clinical parameters.
The clinical parameters are:
Appearance
0 = normal; 1 = unkept fur; 2 = stooped posture; 3 = hair standing up; 4= sunked flanks, visible ribs
Activity level
0 = normal; 1 = small reduction in activity; 2 = large reduction in activity; 3 = immobility
Recovery
0 = recovered from anesthesia; 1 = not recovered from anesthesia
Wound
0 = normal; 1 = inflammation; 2 = red and infected

An animal with a score of 3 or higher in any of the categories or a combined score of 5 will closely monitored and evaluated together with the veterinarian to decide if a humane endpoint should be conducted

In addition weight will be monitored and if the animal loses weight of 10-15% in 2 consecutive days or if the animal loses more than 20% of weight throughout the course of the experiment then the veterinarian of the NIN will be contacted and a decision will be made.

Animals that do not adapt to the head fixed situation will be removed from the experiment after 3 sessions or 5 if there is evidence of continuous improvement.

Indicate the likely incidence.

[redacted]

[redacted]

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned (‘non-recovery’, ‘mild’, ‘moderate’, ‘severe’).

[redacted]

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No > Continue with Section 3: ‘signatures’.

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals will be sacrificed after the procedures to collect brains for post-mortem investigation of brain activity.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Models studied for [redacted] behaviour

- [redacted]
- [redacted]
- [redacted] behavior
- [redacted] behavior

Experimental strategy for each of the emphatic behavioral models

- 3.4.4.1 Measuring behavior and [redacted] in optimized behavior model
- 3.4.4.2 Behavior , [redacted]
- 3.4.4.3 Effects of interventions on [redacted] and behavior
- 3.4.4.4 Effects of interventions on behavior and [redacted]

Go/no go

Go/no go

Go/no go

Development of methodology

- 3.4.4.1 Development/optimizing behavior model
- 3.4.4.1 Development of techniques for measuring [redacted]
- 3.4.4.2 Determine [redacted] Involved in [redacted] behavior
- 3.4.4.2 Development techniques for measuring [redacted]
- 3.4.4.3 Methodological validation of intervention techniques
  - [redacted]
  - [redacted] interventions
  - [redacted] and [redacted]
- 3.4.4.4

AVD-801002015105
Attachment 2

Table 1. Indicates number of mice per appendix and classified by discomfort levels.

Discomfort levels - Mice

Appendix	total per appendix	none-mild	moderate	
3.4.4.1	3740	1108	2632	
3.4.4.2	1200	96	1104	
3.4.4.3	1200	264	936	
3.4.4.4	300	0	300	
Total		1468	4972	**6440**
Percent		22.8	77.2	

Table 2. Indicates number of rats per appendix and classified by discomfort levels.

Discomfort levels - Rats

Appendix	total per appendix	none-mild	moderate	
3.4.4.1	2540	1108	1432	
3.4.4.2	1200	96	1104	
3.4.4.3	1200	264	936	
3.4.4.4	300	0	300	
Total		1468	3772	**5240**
Percent		28.0	72.0	

11 januari 2015

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de bijbehorende toelichting, waarin elke stap in het beoordelingsproces wordt toegelicht

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD/801002015105
2. Titel van het project: Multilevel investigation of the neural correlates of emotional understanding in rodents.
3. Titel van de NTS: Neuronale basis van emotie
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 17-04-2015
   - ✓ aanvraag compleet (herziening): 03-06-2015
   - ✓ in vergadering besproken: 23-04-2015
   - ✓ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en): n.v.t.
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ✓ advies aan CCD: 05-06-2015
7. Eventueel horen van aanvrager
   - Datum: n.v.t.
   - Plaats: n.v.t.
   - Aantal aanwezige DEC-leden: n.v.t.
   - Aanwezige (namens) aanvrager: n.v.t.
8. Correspondentie met de aanvrager:
   - Datum 24-04-2015
   - Strekking: completering van de aanvraag
   - Datum antwoord (gecompleteerde versie): 03-06-2015
   - Strekking van de antwoorden: de aanvraag is gecompleteerd
9. Eventuele adviezen door experts (niet lid van de DEC): geen

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig. Het omvat dierproeven in de zin der wet.
2. De aanvraag betreft een nieuwe aanvraag. Er is enige overlap met een aantal al van een positief advies voorziene DEC-protocollen.
3. De DEC is competent om over deze projectvergunningsaanvraag te adviseren. De benodigde expertise op dit wetenschappelijk terrein is aanwezig binnen de DEC. Geen van de DEC-leden is betrokken bij het betreffende project.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering: n.v.t.

C. Beoordeling (inhoud):

1. Het project is wetenschappelijk verantwoord.

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

3. De doelstelling, in relatie tot de uitvoering, is duidelijk omschreven in de aanvraag; te weten het verkrijgen van fundamenteel wetenschappelijke inzichten in de basale neuronale mechanismen van empathie op verschillende integratieniveaus: moleculair-genetisch, cellulair, hersengebieden en netwerken. Het is aangetoond dat de basale neuronale mechanismen resulterend in de overdraagbaarheid van emoties (empathie), aanwezig zijn in ratten en muizen. Het gebruik van de bestaande proefdiermodellen voor de overdraagbaarheid van emoties en het daaruit resulterende gedrag en de ontwikkeling van nieuwe modellen hiervoor zal een beter inzicht geven in neuronale basis van het empathisch vermogen van de mens.

   Het fundamenteel wetenschappelijke belang acht de DEC substantieel. Het vermogen van een individu om emoties te herkennen bij een ander resulterend in vergelijkbare emoties in de waarnemer zelf en in sociaal gedrag vormt een belangrijke basis het functioneren van een individu in een groep. Het verkrijgen van fundamentele wetenschappelijke kennis van de processen en factoren die ten grondslag liggen aan dit soort gedrag is van belang voor een beter inzicht in het functioneren van de mens als sociaal individu en de mens als onderdeel van de maatschappij waarbinnen een groot belang wordt toegekend aan sociaal gedrag. Er zijn bij de mens grote interindividuele variaties in empathisch en sociaal gedrag en een beter inzicht in hoe dit gedrag tot stand komt van klinisch belang voor ziektebeelden als autisme, antisociale persoonlijkheidsstoornissen en schizofrenie. Het project dient daarmee, op termijn, een belangrijk maatschappelijk belang.

4. De gekozen strategie, experimentele aanpak in combinatie met de infrastructuur

op het Nederlands Herseninstituut en de expertise van de betrokken onderzoeksgroep bieden een realistisch uitzicht op het behalen van de beoogde doelstellingen binnen gevraagde looptijd van het project. Naast de dierexperimentele aanpak is er een lopende onderzoekslijn naar de overdraagbaarheid van emoties en het daaruit resulterende gedrag in de mens, gebruikmakend van fMRI en EEG registraties. Dit onderzoek heeft belangrijke resultaten en publicaties opgeleverd. Het gebruik van invasieve technieken met een hoge temporele en spatiële resolutie om zo inzicht te krijgen in cellulaire processen en inzicht te krijgen in de relatie tussen neuronale activiteit en gedrag is echter niet mogelijk in de mens. Beide onderzoekslijnen zullen naast elkaar worden uitgevoerd met een sterke onderlinge wisselwerking. Hoewel een aantal modellen voor overdraagbaarheid van emoties en het daaruit resulterende gedrag in ratten/muizen beschikbaar is, bevindt een deel van het dierexperimenteel werk zich in een exploratieve fase waarbij gezocht wordt naar goede gedragsmodellen voor verschillende aspecten van dit gedrag.

5. Alle dieren worden gefokt voor het gebruik in dierproeven, er is geen sprake van hergebruik. Het is een noodzakelijk onderdeel van de proeven dat een deel van de dieren gedurende een korte of langere tijd solitair wordt gehuisvest. In die periode kunnen de dieren elkaar wel zien, ruiken en aanraken. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit het wild. De toegepaste methoden voor anesthesie/euthanasie zijn conform de Richtlijn.

6. Het cumulatieve ongerief gepaard gaand met de dierproeven, zoals beschreven in de vier verschillende type dierproeven, is naar inschatting van de DEC, voor het merendeel van de dieren matig (77% van de 6440 muizen en 72% van de 5240 ratten) en voor de overige dieren licht. Deze inschatting van de DEC is in overeenstemming met het niveau van cumulatief ongerief zoals dat is geclassificeerd door de onderzoekers. Dit is gebaseerd op hun ervaring met de gebruikte modellen in vergelijkbare, al uitgevoerde, dierproeven.
   Er moet worden opgemerkt dat in sommige modellen het noodzakelijk is om aversieve stimulaties (bijvoorbeeld korte elektrische schokken) te gebruiken als onderdeel van de gedragstesten en dat pijnbestrijding in deze gevallen niet wordt toegepast omdat dit strijdig is met de doelstelling van het experiment. In alle andere gevallen wordt een adequate pijnbestrijding gebruikt.

7. Binnen het project wordt maximaal gebruik gemaakt van methoden die de voorgestelde dierproeven geheel of gedeeltelijk **vervangen.** Een belangrijk onderdeel van de experimentele strategie is de gefaseerde opzet zoals beschreven in onderdeel 3.4.3 en gevisualiseerd in de bijlage “roadmap”. Eerst wordt een nieuw model en de benodigde methoden om betrouwbare uitleesparameters met een zo laag mogelijke variabiliteit te behalen volledig geoptimaliseerd. Daarna wordt overgegaan tot vervolgexperimenten om gedrag, fysiologie en neuronale activiteit te bestuderen en om interventies toe te passen.

Nieuwe inzichten in de processen en factoren die ten grondslag liggen aan de overdraagbaarheid van emoties en het daaruit resulterende gedrag kunnen op dit moment alleen maar verkregen worden in een intact organisme. Naast de dierexperimentele aanpak wordt er onderzoek gedaan naar empathisch/sociaal gedrag in de mens. Beide onderzoekslijnen zullen naast elkaar worden uitgevoerd met een sterke onderlinge wisselwerking. Gezien het invasieve karakter van het onderzoek naar de basis van empathie gestuurd gedrag op cellulair, moleculair en genetisch niveau kan dit onderzoek niet in proefpersonen worden uitgevoerd. Naar het oordeel van de DEC zijn er geen alternatieven beschikbaar voor het voorgestelde gebruik van intacte dieren om te doelstelling van dit project te realiseren.

8. In het project wordt optimaal tegemoet gekomen aan de vereisten van **vermindering** van dierproeven. De onderzoeksgroep heeft ervaring met dit soort experimenten en door een gefaseerde opzet worden er per experiment niet meer dat het minimum aantal benodigde dieren ingezet. Technieken en procedures worden zorgvuldig toegepast. Het totaal aantal te gebruiken dieren in het project is realistisch geschat.

9. De uitvoering van het project is in overeenstemming met de vereisten van **verfijning** van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.
   Bij de opzet wordt rekening gehouden met dierenwelzijn en wel op de volgende manieren: 1) een uitgebreide gewenningsperiode aan de onderzoeker en de opstelling om stress te verminderen, 2) het gebruik van adequate anesthesie en analgesie waar nodig/mogelijk, 3) een intensieve monitoring van de proefdieren en 4) een sociale huisvesting van de dieren waar mogelijk.

   Er moet worden opgemerkt dat in sommige van de modellen het noodzakelijk is om aversieve stimulaties (bijvoorbeeld korte elektrische schokken) te gebruiken als onderdeel van de gedragstesten en dat pijnbestrijding in deze gevallen niet wordt toegepast. De DEC acht dit onvermijdbaar voor het bereiken van het doel van het onderzoek. In alle andere gevallen wordt een adequate pijnbestrijding gebruikt.
   Daarnaast wordt in sommige proeven een deel van de dieren gedurende een korte of langere tijd solitair gehuisvest. In die periode kunnen de dieren elkaar wel zien, ruiken en aanraken. De DEC acht dit onvermijdbaar voor het bereiken van het doel van het onderzoek.

   Er is geen sprake van belangwekkende milieueffecten.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en is geformuleerd in begrijpelijke taal. De NTS voldoet daarmee aan de eisen zoals gesteld in artikel 10.a.1.7 van de Wod.

D. Ethische afweging

De centrale vraag voor de ethische afweging is of het belang van het doel van dit project opweegt tegen het ongerief dat de dieren ondergaan (geclassificeerd voor het merendeel van de dieren als matig). Het doel van het project is het verkrijgen van fundamenteel wetenschappelijke inzichten in de basale neuronale mechanismen van empathie op verschillende integratieniveaus. Het onderzoek is primair fundamenteel wetenschappelijk van karakter. De verwachting is dat de resultaten van het onderzoek, op termijn, kunnen bijdragen aan een beter inzicht in de oorzaken van verschillende ziektebeelden waarbij empathisch/sociaal gedrag verstoord is. Het project dient daarmee, op termijn, *een belangrijk maatschappelijk belang*.

Het fundamenteel wetenschappelijke onderzoek in dit project is van hoge kwaliteit en het project is uit wetenschappelijk oogpunt verantwoord. De onderzoeksgroep beschikt over ervaring met de gekozen onderzoeksstrategie en met de voorgestelde typen dierproeven. De DEC is van mening dat de resultaten van de dierproeven zullen bijdragen aan het behalen van de geformuleerde doelstellingen en schat de kans op het realiseren van deze doelstellingen in als hoog. De verkregen fundamenteel wetenschappelijke kennis is essentieel om te komen tot een beter begrip van de neuronale basis van naar de overdraagbaarheid van emoties en het daaruit resulterende gedrag en dient daarmee een *substantieel wetenschappelijk belang*.

Bij de het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van vervanging, vermindering en verfijning van de dierproeven. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald.

De DEC komt tot de conclusie dat de doeleinden van het project het voorgestelde gebruik van de proefdieren en het daarmee samenhangende ongerief van de proefdieren rechtvaardigen.

E. Advies

1. Advies aan de CCD
   - ✓ **De DEC adviseert de vergunning te verlenen**
2. Het uitgebrachte advies is gebaseerd op consensus.
3. Er zijn geen knelpunten of dilemma's gesignaleerd tijdens het beoordelen van de aanvraag of het formuleren van het advies.

11

[redacted]

Van: ZBO-CCD
Verzonden: woensdag 17 juni 2015 15:10
Aan: [redacted]
CC: [redacted]
Onderwerp: Aanvullende informatie AVD801002015105
Bijlagen: Aanvraag aanvullende informatie AVD801002015105.pdf

Dear [redacted],

Attached you can find an official letter requesting extra information we need to evaluate your project application. You can send us the answers in English.

The letter is also mailed to the establishment licence holder, [redacted]

Met vriendelijke groet,

[redacted]

Uitvoeringsexpert

Centrale Commissie Dierproeven www.centralecommissiedierproeven.nl
Postbus 20401 | 2500 EK | Den Haag
T: 0900 2800028
E: info@zbo-ccd.nl

12

[redacted]

Van: ZBO-CCD
Verzonden: vrijdag 12 juni 2015 11:35
Aan: [redacted]
CC: [redacted]
Onderwerp: Aanvulling aanvraag AVD801002015105
Bijlagen: Aanvulling aanvraag AVD801002015105.pdf

Geachte [redacted]

Bijgaand de aanvullingsbrief voor aanvraag AVD801002015105 die vandaag ook per post is verzonden aan [redacted]

De leges die u verschuldigd bent zijn nog niet door ons ontvangen of de betaling is nog niet verwerkt. Zoals in de factuur staat, moeten de leges binnen 30 dagen door ons zijn ontvangen. Uw aanvraag is niet compleet als de leges niet zijn ontvangen.

Met vriendelijke groet,

Centrale Commissie Dierproeven www.centralecommissiedierproeven.nl
Postbus 20401 | 2500 EK | Den Haag
T: 0900 2800028
E: info@zbo-ccd.nl

13

[redacted]

Van: [redacted]
Verzonden: dinsdag 16 juni 2015 8:57
Aan: ZBO-CCD
Onderwerp: aanvulling AVD801002015105
Bijlagen: 15-06-16 PD, aanvr. aanvulling projectaanvraag DP AVD801002015105.pdf; Ontvangstbevestiging en factuur.pdf

Categorieën: Dossierhouder: [redacted]

Geachte CCD,

In antwoord op uw verzoek vandaag door ons ontvangen over de leges van AVD801002015105. Volgens mijn informatie wordt morgen de betalingsopdracht verstuurd door het betrokken instituut.

Ik moet u ook attent maken op het feit dat in de ontvangstbevestiging, waarin ook de factuur is opgenomen, als **vervaldatum voor de betaling 5 juli** wordt genoemd. De tekst van die brief luidt ´Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld". De genoemde vervaldatum is nog niet verstreken terwijl ik uit uw brief van 12 juni (7 dagen na indiening!) begrijp dat "de behandeling van uw aanvraag wordt opgeschort tot het moment dat uw aanvraag compleet is" d.w.z. de leges zijn voldaan. Het bericht is automatisch gegenereerd maar timing en tekst dient wellicht aanpassing.

Met vriendelijke groet,
[redacted]
DEC-KNAW

14

[redacted]

Van: [redacted]
Verzonden: woensdag 17 juni 2015 13:47
Aan: ZBO-CCD
Onderwerp: RE: Aanvullende vragen aanvraag AVD/801002015105 t.a.v [redacted]

Categorieën: Dossierhouder: [redacted]

Geachte [redacted]

Ik heb uw vragen bestudeerd. De vragen lijken deels betrekking te hebben op inhoudelijke aspecten van de aanvraag en deels betrekking te hebben op de discussie binnen de DEC en het advies. Dit veroorzaakt m.i. een wat onduidelijke situatie welke vragen voor de onderzoeker zijn en welke voor de DEC. Ik stel voor dat u alle vragen direct aan de onderzoeker stuurt met mogelijkerwijs een herformulering van de vragen 6 en 7. *Ik moet u er op attent maken dat de indiener geen Nederlands spreekt*.

Mocht u specifieke vragen over het advies hebben dan ontvang ik die graag van u.

Ik hoor graag uw reactie.

Met vriendelijke groet,
[redacted] DEC-KNAW

From: ZBO-CCD [mailto:ZBO-CCD@minez.nl]
Sent: Tuesday, June 16, 2015 2:51 PM
To: secretariaat DEC
Subject: Aanvullende vragen aanvraag AVD/801002015105

Geachte [redacted]

We zijn bezig met het beoordelen van de aanvraag AVD801002015105 met titel “Multilevel investigation of the neural correlates of emotional understanding in rodents.” Om deze aanvraag te kunnen beoordelen hebben wij aanvullende informatie nodig. Graag vernemen we van u of deze vragen in de DEC-vergadering besproken zijn en zo ja: welke de antwoorden zijn.

Dierproef 3.4.4.1:
1) De noodzakelijkheid om de beschreven hoeveelheid stammen/lijnen te gebruiken om een test te valideren besproken waarbij niet helemaal helder is wat de opbrengsten daarvan zijn.
2) Het doel van het fokken van dieren met bepaalde karakteristieken en op basis waarvan een selectie wordt gemaakt. Er wordt beschreven dat maar 10% van de nakomelingen wordt gebruikt voor het genereren van 5 nieuwe generaties, waarbij niet duidelijk is wat er met deze dieren gebeurt.
3) Ziet u dit als het genereren van nieuwe lijnen, waarvoor een welzijnsevaluatie gedaan moet worden zodra de lijnen established zijn?
4) De onderzoeker schrijft van plan te zijn om drie positieve en drie negatieve emoties te testen, maar ook dat de meerderheid van de dieren in maximaal drie gedragstesten zal worden gebruikt. Wordt hier bedoeld dat elke dier mee doet aan maximaal drie verschillenden positieve/negatieve emoties of drie testen voor dezelfde emotie? Uit het aantal gevraagde dieren is niet helder op te maken wat hier bedoeld wordt.

Alle dierproeven:
5) Op basis van welke criteria wordt er al dan niet gekozen voor de ‘optionele’ behandelingen vermeld in de aanvraag.

6) Is het gebruik van beide geslachten in alle dierproeven besproken? Zo ja: wat is het advies van de DEC? Zo neen: waarom niet?
7) Heeft de DEC de mogelijkheid besproken om enkele dierproeven te combineren zodat dezelfde dieren gebruikt kunnen worden? Dit in het kader van vermindering en verfijning.

Indien deze vragen niet in de DEC-vergadering besproken zijn geweest en u de antwoorden niet kent, willen we deze vragen rechtstreeks aan de onderzoeker stellen. We vragen u om uiterlijk morgen, 17 juni 2015, om 14:00 op deze email te reageren, gezien wij de klok alleen stil kunnen zetten als de vragen aan de onderzoeker worden gesteld.

Alvast hartelijk dank voor uw medewerking.

Met vriendelijke groet,

[redacted]

Uitvoeringsexpert

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E: info@zbo-ccd.nl

15

[redacted]

Van: Info-zbo
Verzonden: donderdag 16 juli 2015 9:22
Aan: [redacted]
Onderwerp: Terugkoppeling aanvraag AVD801002015105

Geachte heer [redacted]

Op 5 juni 2015 heeft de DEC KNAW advies uitgebracht aan de CCD betreffende het project " Multilevel investigation of the neural correlates of emotion understanding in rodents", met aanvraagnummer AVD801002015105.

De CCD heeft nog enkele vraag aan de aanvrager gesteld, die u ook hebt ontvangen.

De aanvrager heeft een goed onderbouwd antwoord gegeven.

De CCD heeft besloten de vergunning, overeenkomstig uw advies, te verlenen onder de volgende voorwaarden:

De onderzoeker zal zowel de go/no-go momenten als de criteria om een optionele behandeling uit te voeren met de IvD afstemmen.

Daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen zullen de dieren en experimenten na het verlenen van de vergunning formeel onder deze vergunning gaan vallen, zoals u in uw projectvoorstel ook heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

In Artikel 10, eerste lid, onder a, Wet op de dierproeven, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

De aanvrager en verantwoordelijk onderzoeker zijn hierover ingelicht.

We hopen u hiermee voldoende te hebben geïnformeerd.

Met vriendelijke groet,

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**
Postbus 20401 | 2500 EK | Den Haag
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E: info@zbo-ccd.nl

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Master the mind

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IBAN: NL33 DEUT 0546 9000 54
BIC: DEUTNL2N

Reference
Phone [redacted]
E-mail [redacted]
Date June 30-2015
Subject Reply to additional questions regarding our application

Dear [redacted]

Deze brief schrijven wij n.a.v. uw brief d.d. 17 juni 2015. In uw brief vraagt u om aanvullende informatie over de projectvergunningaanvraagdierproeven (AVD801002015-105). Hierbij voldoen wij aan dit verzoek. In het navolgende herhalen we eerst de in het Nederlands gestelde vraag met daarop volgend de beantwoording van de vraag in het Engels.

1) In uw aanvraag schrijft u van plan te zijn om verschillende stammen en lijnen [redacted]. Zou u kunnen toelichten waarom is het noodzakelijk [redacted] om een test te valideren en in hoeverre de verschillen tussen de [redacted] invloed kunnen hebben op de te valideren testen?

For the *validation* of a behavioral paradigm [redacted]. Only when a test is completely validated [redacted] experimental variables that will be examined to elucidate [redacted] implicated in the modulation of behavior. For example, researchers have shown that [redacted] and show significantly [redacted]. As a strategy, we first validate the behavioral test and we then examine the factors that affect the behavior [redacted]. Thus, the differences that we encounter between [redacted] will not have an influence in the validation of the behavioral tests.

2) Als onderdeel van uw dierproef bent u van plan om dieren met [redacted]. We zullen graag meer informatie van u willen ontvangen over het doel van deze proef, de karakteristieken die [redacted] en de manier [redacted]. Kan deze proef leiden tot een [redacted] en/ of kunnen de [redacted] hebben? [redacted]

The idea of using [redacted] behavioral traits, based on endogenous natural variability of the expression of beh[redacted] [redacted] our [redacted]. For example, we would [redacted]

3) Onder het [redacted] nodig voor de gedragstesten schrijft u [redacted] [redacted] u kunnen [redacted] zal worden gebruikt. Bedoelt u hier [redacted] [redacted]. All our tests are done in [redacted] l purposes), [redacted] developing the behavioral paradigm. [redacted] The statement in appendix 1, [redacted]

4) In de beschrijving van de dierproeven schrijft u dat een groot deel van de behandelingen optioneel zijn. Zou u kunnen uitleggen op basis waarvan u al dan niet kiest voor die behandelingen?

The optional criteria has been added to indicate that a certain [redacted] through the option described. The options that have been included are based on our experience with current experimental results and observations. For example, we recently noticed that [redacted]. Thus, it might be important to measure [redacted] Given that we know that [redacted], but we also don't need to measure it in every single experiment/animal we left it as a possible procedure to be performed which will depend on the experimental question. Before the start of each experiment the exact procedure and combinations will be submitted to the IvD for evaluation.

5) U bent van plan om mannelijke en vrouwelijke dieren te gebruiken? Geldt dit voor alle experimenten?

Our experience so far has taught us that there is a difference in the behavior between males

and females and i [redacted] nales is therefore preferred in order to reduce the variability as much as possible. [redacted] he [redacted] facto [redacted] nvestigating.

[redacted] en tot minder gebruikte dieren?

[redacted] First, [redacted] own, but if we want to understand the [redacted] d outcome and will allow us to draw robust scientific conclusions. Second, because [redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
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1000GC Amsterdam

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2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-2800028 (10 ct /min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015105

Datum 14 juli 2015
Betreft Beslissing aanvraag projectvergunning dierproeven

Bijlagen
1

Geachte [redacted]

Op 5 juni 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Multilevel investigation of the neural correlates of emotion understanding in rodents' met aanvraagnummer AVD801002015105. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de dierproeven (hierna de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. U kunt met uw project 'Multilevel investigation of the neural correlates of emotion understanding in rodents' starten. De vergunning wordt afgegeven van 14 juli 2015 tot en met 1 juli 2020.

Procedure
Bij uw aanvraag heeft u een advies van de dierexperimentencommissie DEC KNAW gevoegd. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering.
Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze:

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163.

Bijlagen
- Vergunning
  - Hiervan deeluitmakend:
    - DEC-advies
    - Weergave wet en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: KNAW
Adres: Postbus 19121
Postcode en woonplaats: 1000 GC Amsterdam
Deelnemersnummer: 80100

deze projectvergunning voor het tijdvak 14 juli 2015 tot en met 1 juli 2020, voor het project 'Multilevel investigation of the neural correlates of emotion understanding in rodents' met aanvraagnummer AVD801002015105, volgens advies van Dierexperimentencommissie DEC KNAW.
De functie van de verantwoordelijke onderzoeker is groepsleider.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen bij digitale indiening op 5 juni 2015;
2. de bij het aanvraagformulier behorende bijlagen:
    a. Projectvoorstel, zoals ontvangen bij digitale indiening op 5 juni 2015;
    b. Niet-Technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 5 juni 2015;
    c. Advies van dierexperimentencommissie DEC KNAW d.d. 5 juni 2015 en ontvangen op 5 juni 2015;
    d. De aanvullingen op uw aanvraag, ontvangen op 2 juli 2015.

Dierproeven

Naam dierproef	**Diersoort**	**Aantal dieren**	**Ernst**
Measurement of social, prosocial and antisocial behaviour	Muizen (*Mus musculus*) Ratten (*Ratus norvegicus*)	1108 2632 1108 1432	Licht Matig Licht Matig
Measure activity at the neuronal, brain region or network level in different behavioural paradigms (behaviour + neuronal activity measurement)	Muizen (*Mus musculus*) Ratten (*Ratus norvegicus*)	96 1104 96 1104	Licht Matig Licht Matig
Manipulate/alter and then measure the behavioural consequences in different behavioural paradigms	Muizen (*Mus musculus*) Ratten (*Ratus norvegicus*)	264 936 264 936	Licht Matig Licht Matig
Manipulate/alter and then measure the consequences in terms of activity at the neuronal, brain region or network level in different behavioural paradigms	Muizen (*Mus musculus*) Ratten (*Ratus norvegicus*)	300 300	Matig Matig

Voorwaarde:
Op grond van artikel 10a1 lid 2 Wet zijn aan een projectvergunning voorwaarden te stellen:

De onderzoeker zal zowel de go/no-go momenten als de criteria om een optionele behandeling uit te voeren met de IvD afstemmen.

Daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen zullen de dieren en experimenten na het verlenen van de vergunning formeel onder deze vergunning gaan vallen, zoals u in uw projectvoorstel ook heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

In Artikel 10, eerste lid, onder a, Wet op de dierproeven, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Weergave wet- en regelgeving

Dit project en wijzigingen

Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging

De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving

In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef

Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven

ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.

20

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.zbo-ccd.nl

T 0900 28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015105

Bijlagen
2

Datum 5 juni 2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 5 juni 2015.
Het aanvraagnummer dat wij hieraan hebben gegeven is AVD801002015105
Gebruik dit nummer als u contact met ons opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te betalen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Datum
5 juni 2015

Onze referentie
Aanvraagnummer
AVD801002015105

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:

- Gegevens aanvraagformulier
- Factuur

Checklist bijlagen

Verplichte bijlagen:

[x] Projectvoorstel
[x] Beschrijving Dierproeven
[x] Niet-technische samenvatting

Overige bijlagen:

[x] Melding Machtiging
[x] DEC-advies

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.zbo-ccd.nl

T 0900 28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015105

Factuurdatum	5 juni 2015
Vervaldatum	5 juli 2015
Factuurnummer	201570105
Betreft	Factuur Aanvraag projectvergunning dierproeven

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD801002015105	€ 741,-

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015105

Bijlagen

Datum 2 juli 2015
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 5 juni 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project “Multilevel investigation of the neural correlates of emotion undestanding in rodents” met aanvraagnummer AVD801002015105. Op 17 juli 2015 hebben wij u om aanvullende informatie gevraagd. Deze informatie hebben wij nog niet ontvangen.

Welke informatie nog nodig
Wij hebben de volgende informatie van u nodig om uw aanvraag te kunnen beoordelen:

Dierproef 3.4.4.1

1) In uw aanvraag schrijft u van plan te zijn om verschillende stammen en lijnen ratten en muizen te testen. Zou u kunnen toelichten waarom is het noodzakelijk om meerdere stammen/lijnen ratten en muizen te gebruiken om een test te valideren en in hoeverre de verschillen tussen de stammen/lijnen invloed kunnen hebben op de te valideren testen?

2) Als onderdeel van uw dierproef bent u van plan om dieren met bepaalde karakteristieken te fokken. We zullen graag meer informatie van u willen ontvangen over het doel van deze proef, de karakteristieken die gekozen worden en de manier waarop dat gebeurt. Kan deze proef leiden tot een nieuwe stam en/ of kunnen de nakomelingen ongerief hebben? Wat gebeurt er met de nakomelingen?

3) Onder het aantal dieren nodig voor de gedragstesten schrijft u dat er 1600 ratten en 1600 muizen nodig zijn om 3 positieve en 3 negatieve emoties te testen en ook voor het testen van de instrumentele agressie. Voor elk gedrag meldt u 200 dieren nodig te hebben. Zou u kunnen uitleggen hoeveel groepen bent u van plan te gebruiken? Daarnaast schrijft u dat de meerderheid van de dieren in maximaal drie proeven zal worden gebruikt. Bedoelt u hier dat elk dier in maximaal drie gedragstesten per emotie wordt getest of in maximaal drie verschillende positieve/negatieve emoties?

Alle dierproeven:

4) In de beschrijving van de dierproeven schrijft u dat een groot deel van de behandelingen optioneel zijn. Zou u kunnen uitleggen op basis waarvan u al dan niet kiest voor die behandelingen?

5) U bent van plan om mannelijke en vrouwelijke dieren te gebruiken? Geldt dit voor alle experimenten?

6) In hoeverre zou het mogelijk zijn om enkele dierproeven te combineren, om in dezelfde dieren de neuronale activiteit op basisniveau te onderzoeken alsook te manipuleren? Zou dat niet betrouwbaardere resultaten en minder variatie kunnen opleveren, alsook leiden tot minder gebruikte dieren?

Opsturen binnen zeven dagen
Wij willen u de mogelijkheid bieden alsnog aan ons verzoek te voldoen. Dit kan door binnen zeven dagen na de datum van deze brief de gegevens te sturen. U kunt dit aanleveren via NetFTP. Wanneer wij de aanvullende informatie niet binnen de gestelde termijn hebben ontvangen, kunnen wij uw aanvraag buiten behandeling stellen.

Wanneer een beslissing
De behandeling van uw aanvraag is opgeschort tot het moment dat uw aanvraag compleet is. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:
- formulier Melding Bijlagen via de post

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

- U wilt één of meerdere bijlagen naar ons versturen? Voeg *altijd* deze Melding Bijlagen toe. Wij weten dan welke documenten van u zijn en hoeveel documenten u opstuurt.
- Meer informatie vindt u op www.centralecommissiedierproeven.nl
- Of bel met ons: 0900 28 000 28 (10 ct/min).

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

1.2 Bij welke aanvraag hoort de bijlage?
Het aanvraagnummer staat in de brief of de ontvangstbevestiging.

Aanvraagnummer	

2 Bijlagen

2.1 Welke bijlagen stuurt u mee?
Vul de naam of omschrijving van de bijlage in.

☐	
☐	
☐	

3 Ondertekening

3.1 Onderteken het formulier en stuur het met alle bijlagen op naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

	Inventaris Wob-verzoek W16-01								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 2015125								
1	Aanvraagformulier				x		x	x	
2	Niet-technische samenvatting	x							
3	Projectvoorstel				x			x	
4	Flow chart			x					
5	Bijlage beschrijving dierproeven 1			x					
6	Bijlage beschrijving dierproeven 2			x					
7	Bijlage beschrijving dierproeven 3			x					
8	Bijlage beschrijving dierproeven 4				x			x	
9	Bijlage beschrijving dierproeven 5			x					
10	Bijlage beschrijving dierproeven 6			x					
11	Bijlage beschrijving dierproeven 7			x					
12	Overzicht aantallen			x					
13	Ontvangstbevestiging				x		x	x	
14	DEC-advies				x		x	x	
15	Mail aanvraag 22-6-2015				x		x	x	
16	Mail aanvullende informatie				x		x	x	
17	Aanvullende informatie				x		x	x	
18	Beschikking en vergunning				x		x	x	
19	Advies CCD		x						x

22 JUNI 2015

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☑ Ja > *Vul uw deelnemernummer in*	80102 (Hubrecht Instituut-KNAW) 80101 NIN
☐ Nee > *U kunt geen aanvraag doen*	

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	KNAW
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	5 4 6 6 7 0 8 9

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	
Postbus	Postbus 19121
Postcode en plaats	1000GC Amsterdam
IBAN	NL94
Tenaamstelling van het rekeningnummer	Hubrecht Instituut / Nederlandshersen Instituut

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	Group Leader
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	☑ Dhr. ☐ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☑ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☑ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☑ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	0 1 _0 6 _ 2 0 1 5
		Einddatum	0 1 _0 6 _ 2 0 2 0
3.2	Wat is de titel van het project?	The molecular and cellular mechanisms of tumor initiation, growth, metastasis and thera	
3.3	Wat is de titel van de niet-technische samenvatting?	Het begrijpen van het ontstaan en ontwikkeling van kanker, uitzaaiingen en resistentie t	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC-KNAW
		Postadres	[redacted] Amsterdam
		E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- [x] Nieuwe aanvraag Projectvergunning € 741,00 Lege
- [ ] Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

- [ ] Via een eenmalige incasso
- [x] Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- [x] Projectvoorstel
- [x] Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- [x] flow chart
- [ ] table with experimental groups

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted]
Plaats	Amsterdam
Datum	0 1 - 0 6 - 2 0 1 5
Handtekening	[redacted]

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	KNAW
1.3	Provide the title of the project.	The molecular and cellular mechanisms of tumor initiation, growth, metastasis and therapy resistance

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Cancer is one of the most deadly diseases in the western-world. When diagnosed early, a primary tumor can be surgically removed, and most patients can be cured at this early stage. However when cancer is diagnosed at a later stage, there is the chance that cells from the primary tumor have detached, spread to other sites in the body, and have

formed new distant tumor sites. At this stage cancer is far more difficult to treat and most patients die as a consequence of complications resulting from metastasis.
Upon genetic mutations, tumor cells “high-jack” cellular processes, that under non-disease circumstances, only takes place in different cell types or in specific phases of development. Examples of cellular processes that cancer cells “high-jack” include cell division and cell growth, a change from an epithelial cell state to a mesenchymal cell state (known as epithelial-to-mesenchymal transition (EMT)), and enhanced cell motility. The micro-environment (the environment that directly surround the mutated cell) and the macro environment (the whole tumor/animal) is an additional driver for picking the processes that are “high-jacked” by cancer cells. The acquisition of these molecular and cellular processes drives tumor initiation, growth, and metastasis.
There are many different cellular processes that can be “high-jacked” leading to cancer or leading to the progression of cancer. Different tumor types (e.g. breast vs colon) but even various variants of the same tumor type can have different genetic mutations and micro- and macro-environments, and therefore can adapt a different set of molecular and cellular processes that they up- or down-regulate. This also means that e.g. breast tumor cells behave differently than colorectal tumor cells, and that these tumor types need to be treated as two independent diseases that need to be investigated independently in their natural orthotropic environment in the context of a whole animal. This type of knowledge is key for the design of therapies that are tailor-made for every patient (personalized medicine) that aim to target the specific processes acquired by the tumor cells in that particular patient.
Tumors are extremely heterogeneous and consist of a variety of tumor cells that have “high-jacked” different processes due to a variation in mutations and environments that the cells experience. Sometimes, just a few individual cells high-jack cellular processes important for e.g. metastasis, while all the other cells have not acquired these traits. The behavior of these few dangerous cells cannot be studied with traditional techniques including histochemistry, (q)PCR and western blotting, since they provide a snapshot of a large population of cells and lack crucial information on the history of these few individual cells. The [redacted] lab has developed unique [redacted] to visualize and study the behavior of individual cells in living mice. Using these techniques, the [redacted] lab can identify and characterize the dynamic behavior of individual cells that are responsible many of the key processes of cancer. With these techniques, we provide unique insights in the molecular and cellular processes that play a role in tumor initiation, tumor progression, metastasis, and the development of therapy resistance. With this knowledge we have the ultimate aim to contribute to the improvement cancer diagnosis, cancer prevention, and cancer treatment in human patients.
Below it will be explained why it is important to study processes required for tissue homeostasis and development in the initiation of a tumor, how tumors develop and progress to a metastatic state and how the micro- and macro-environment is key in these processes.

1) Tumor initiation:
As already indicated above, for the initiation and progression of a cancer, multiple genetic modifications have to occur in the same single cell leading to “high-jacking” of processes specific for other cell types or developmental processes. It has been speculated that long-living cells are more likely to accumulate genetic lesions than short-living cells. In particular adult stem cells, that are located in a special microenvironment (stem cell niche) that provides cues to self-renew, are long-lived and are the source for all differentiated cells in the tissue. Therefore, it is hypothesized that accumulation of genetic oncogenic alterations in stem cells initiates neoplastic growth. Indeed, deletion of the tumor suppressor gene APC in intestinal stem cells leads to adenoma formation in the small intestine while deletion of this gene in differentiated cells does not. Recently, the idea that stem cells represent a static and long-living population of cells has been disputed. For example, we have developed a novel approach for continuous [redacted] stem cells in the gut ([redacted]), and showed that stem cells compete for the stem cell niche, so that the progeny of one stem cell may outcompete (all) other stem cells. Moreover, we showed that more differentiated cells can still revert back to a stem cell state when they enter the stem cell niche. Based on this it has been hypothesized that due to stem cell competition, stem cells with tumor-inducing mutations can be replaced by intact stem cells, thereby protecting tissues from initiating tumors when mutations are acquired. Therefore, the knowledge obtained from this type of experiments is important to fully understand how stem cells can accumulate mutations that are required for the initiation of cancer.

2) Cancer progression.
It is hypothesized that cell hierarchy (where just a few cells with stem cell properties drive growth) may also exist in tumors, referred to as the cancer stem cell hypothesis. However, both the actual existence of cancer stem cells as well as the similarities between cancer stem cells and (tissue) stem cells in other tissues are subject to scientific debate. Nevertheless, if correct, these cancer stem cells may also be long-lived (compared to their differentiated counterparts). Therefore these cells may also be susceptible for the accumulation of mutations that are required for a tumor to progress into a metastatic phenotype. These cancer stem cells may appear to be the driving forces of growth of the primary tumor and the metastases. Therefore, the cells that escape from the primary tumor and form distant metastasis should either be a cancer stem cell or cells that temporally “high-jack” these traits. To fully understand how tumors are growing and the state that cells should adapt to metastasize, it is of utmost importance to study how cancer stem cells behave during cancer progression.

3) The tumor microenvironment:
An expanding body of work shows that in addition to the intrinsic characteristics of tumor cells, the tumor microenvironment is a key determinant for angiogenesis (formation of blood vessels), growth, differentiation or

metastasis. The microenvironment of a tumor consists of at least four broad categories of factors including, 1) tumor cells, 2) non-tumor cells (e.g. myeloid, lymphocytes, fibroblasts), 3) secreted soluble factors, 4) non-cellular structural factors (e.g. extracellular matrix (ECM)). Tumor microenvironments are spatially and temporally diverse, and may direct different tumor behaviors. To fully comprehend tumor initiation and progression, it is key to study also the microenvironment that drives cellular behavior.

Research of our lab:
Over the years, we have significantly contributed to a better fundamental understanding of cancer initiation and progression. Our current research focused on the following questions: 1) how is cancer initiated, 2) how do tumors grow and progress, 3) how do tumors metastasize, 4) how become tumors resistant to therapy

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

Over the years, we have developed state-of-the art intravital imaging techniques that enable us to study the dynamic behavior of individual tumor cells that have "high-jacked" different cellular processes than the tumor cells that surrounds them. The visualization of tissues with subcellular resolution in living mice gives us the ability to study individual cells and the ability to study the dynamic aspects of cancer that cannot be studied by any other means. Using our unique tools, it is our ultimate aim is to better understand how cancer is initiated, how cancer progresses and use this knowledge as a starting point on how cancer can be best treated in order to open avenues for the development of new and better cancer treatments.
Our intravital imaging studies focus on the following key questions:

1) What are the molecular and cellular processes important for tissue development and homeostasis, and what goes wrong when tumor growth is initiated?
2) What are the molecular and cellular mechanisms processes that play a role in tumor growth and progression?
3) What are the molecular and cellular mechanisms that play a role in the initiation and development of metastasis?
4) What goes wrong when current clinical strategies fail (therapy resistance, adverse effects of tumor injury), and how to improve this?

There are several reasons why we think that we can achieve our aims:
Our group is embedded in [redacted], which is a center-of-excellence on developmental biology, and stem cell and cancer research. [redacted] provides core facilities for various high-end techniques such as deep sequencing, histology, fluorescent imaging, mRNA expression array, flow cytometry. Moreover, the Hubrecht Institute has just renovated their animal facility, and now it can compete with the best animal facilities that can be found internationally. Dedicated staff provides the regular housing of the animals and support and counsels the scientist in their experiments. Within our group, we have one dedicated and very experienced scientist that overlooks all breeding of mouse lines, experiments and procedures, and trains new people when required. This guarantees that only trained and experienced people perform experiments.
Our research and experiments are constantly evaluated within our group, and by various other groups within our institute and campus. Moreover, our research is positively judged by national and international funding agencies including the [redacted]. Moreover, our group is member of [redacted], which is a consortium of prominent cancer research groups from seven research institutions in the Netherlands. Our ambition is to significantly improve life expectancy and quality of life for cancer patients and to provide multidisciplinary training for the next generation of cancer researchers and specialists. The scientists working in our group are selected based on their excellence and their commitment to the mission of the program.
Over the last few years, we have built up a repertoire on of state-of-the-art [redacted] techniques to study the molecular and cellular aspects of cancer initiation and cancer progression, and cancer treatment in a unique way. In addition a wide range of new reporter mice have been generated and used. This has led to many new discoveries and breakthroughs published in high ranked journals (e.g. Nature and Cell) and our research has been awarded with international prices (e.g. stem cell young investigator award). Our research is funded by all major funding agencies, and received the most prestigious grants (e.g. ERC consolidator).
Our embedment in an excellent scientific environment, our unique techniques and approaches, and our previous achievement makes it very likely that with the experiments described in this project we will make large contributions to our main research questions.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

Tumors are extremely heterogeneous where the individual cells can “high-jack” molecular and cellular processes that the original healthy cell did not have yet. Our research, and especially our intravital imaging technique, has the unique potential to study these individual cells to reveal the molecular and cellular processes that are acquired and the effect it has on their behavior. Therefore, it is expected that this work will provide totally new insights how cancer is initiated, how cancer progresses, how tumors metastasize, and why current treatments are not sufficient/optimal. This fundamental knowledge is required for the development of novel and/or improved therapies against cancer. Moreover, in addition to the field of cancer, this fundamental knowledge is also important for other fields including fundamental cell biology.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

For our overall design of the project, see the flow Chart in attachment 1.

Based on data from previous experiments and available literature, we generate a hypothesis or question about the molecular and/or cellular mechanism of initiation, progression or therapy resistance of cancer. The questions/hypothesis will first be carefully tested on patient material by e.g. immunohistochemistry. For example, we ask whether cells in the tumor can adapt a mesenchymal state in addition to the epithelial state (EMT). Although immunostaining can reveal the existence of cells with these types of states, it only draws a static picture of tumors and can, for example, not show whether epithelial cells adapt only temporally a mesenchymal state. When we have these types of questions/hypotheses that cannot be answered in human material alone, experiments will be designed in cell lines and/or organoids (3D cultures of human or mouse primary cells). For example, we can test whether epithelial cells can temporally adapt a mesenchymal state by exposing these cells to growth factors. As explained in the background, in vitro conditions lack the full complexity of the in vivo environment, and therefore do not tell the full story. For example these experiments do not show whether the temporal mesenchymal state is crucial for successful metastasis when cells need to adapt to a new microenvironment. To answer these kinds of questions mouse experiments will be considered. We will set up optimal breeding schemes to minimize the number of mice to get the correct complex genotype. Moreover, during the experiments mice will be monitored extensively to detect and avoid unnecessary discomfort. For experiments, existing mouse lines will be used, and if required, we will generate a new mouse line(s).

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

See also the flow chart in attachment 1. In the red boxes the choice of animals (A to C), interventions (I to IV) and readout (1 to 3) is indicated and which is described below.

When we have decided to perform an animal experiment, the experiment is design is based on three choices:
A) Choice of mouse model
B) Choice of type of intervention
C) Choice of readout and end-point of the experiment

The considerations of the choices will be explained in more detail below.

For research questions 1, we need to compare normal tissue homeostasis/development with tumor-initiating tissue homeostasis/development. For research question 2 to 4 (see 3.2 purpose), mice need to develop tumors. For 70% of these experiments, tumors are induced (overexpression of oncogenes, or depletion of tumor suppressor genes) and 30% by transplanting neoplastic tissues or cells. As experimental read out, we analyze tissues ex vivo or by intravital microscopy. At the end point of intravital microscopy, tissues will always be analyzed ex vivo to reduce the number of mice required for 3.4.4.1. These experiments are described in the appendices 3.4.4.1. 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5. and 3.4.4.6.

All experiments described in appendices 3.4.4.1 to 3.4.4.6 can provide knowledge on the key questions 1 to 4 (see 3.2.), and the decision route in the flow chart is determined by e.g. the studied tumor model. An example of a typical experiment is to study the migration behavior of cancer cells that have “high-jacked” stem cell properties. For breast tumors, we will choose a mouse model in which an oncogene is overexpressed (e.g. Choice B II for intervention to

overexpress PyMT, and choice C 2 for read-out). To answer the same question for colorectal tumors, a different decision route is required. In this case, the tumor suppressor gene APC depletion leads to tumor formation throughout the colon and subsequently to a non-functional intestine, and a human end point is reached before the tumor progresses to a stage where tumor cells have acquired stem cell properties. In this case, a small piece of colon from APC depleted mice will be transplanted into a recipient mouse (choice B III) so that only one tumor is formed leaving the intestine functional. Since the mouse does not reach the human end point at early stages of tumor progression, the tumor can progress to a stage where tumor cells can “high-jack” stem cell properties and the measurement can be done (choice C2). Thus for the same research questions, different decision routes need to be taken depending on the tumor model and required intervention.

Generation of GM mice (appendix 3.4.4.7):
We will generate new mouse line(s) via standard oocyte injection, blastocyst injection, or via the Crispr/Cas9 system. The Crispr/Cas9 system will especially be used as highly efficient tool for simultaneously multi-gene editing. This prevents the generation and breeding of multiple homozygotes from individually targeted ES cells (Reduction of the 3Rs).

Next, we will identify a possible hampered phenotype in novel (compound) mouse models according to the Consensus document on genetically altered animals. Therefore new transgenic lines and/or KO lines generated via classical methods and/or novel combinations and used for breeding of these aforementioned lines will be monitored for 2 generations to determine the absence or presence of constitutional discomfort.

For some transplantation experiments (e.g. when human organoids are transplanted), immune deficient acceptor mice are required. We breed our own immune deficient NOD-SCID mice. For this, we have 6-10 breeding pairs that will be replaced every 6 month that leads to offspring that will be used for experiments. According to the working document on genetically altered animals of the National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes (corrigendum of 24 Jan. 2013) breeding of immune deficient mice even when kept under proper barrier conditions is considered an experiment and the breeding of these animals is described in 3.4.4.7.

A. Mouse models:

There are several considerations in choosing an animal model:
- GMM that expressed a fluorescent marker in a specific cell type/lineage
- GMM that changes expression of a functional gene(transgene knockout)
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse
- Wt

The choice will be on the following considerations:
- Type of tumor: Tumor cells can “high-jack” a wide variety of molecular and cellular processes, and not one tumor is alike. Therefore it is important to investigate different tumor types and various variants of the same tumor type to reveal whether the acquisition of a particular molecular and cellular process is a general or specific phenomenon (personalized medicine).
- Readout parameters

If the required GMM is not available, than we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. Interventions:

Apart of I) ‘no intervention’ we will use the following additional groups of interventions: II) upregulation or reduction cell types or expression of genes by genetic approaches, drugs or injury, III) transplantation of tissue or cells, and IV) a combination of intervention II and III in the same animal.

I) No experimental interventions (3.4.4.1 and 3.4.4.2):
To visualize for example how stem cells behave during tissue development, we analyze ex vivo (3.4.4.1) or intravital image (3.4.4.2) reporter mice in which e.g. stem cells are labelled with GFP.

II) Cell type specific gene (in)activation and (over)/(mis)expression (3.4.4.1 and 3.4.4.2):
To identify for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating these processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which the gene(s) will be

activated, inactivated, overexpressed and/or misexpressed.

In contrast to the conventional gene-targeting strategy, the use of e.g. the Cre/LoxP recombination system in conjunction with gene targeting has greatly expanded the versatility and avenues with which biologic questions can be addressed in the mouse. This system allows us, by strategically incorporating Cre recombinase recognition (LoxP) sites into the genome and the subsequent expression of the Cre recombinase, to study the consequence of specific ablation, activation and/or over/misexpression of a specific protein. In particular, when Cre is expressed in mice harboring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell), pathogenesis (e.g. cancer cells) of the mouse depending on the specificity and timing of recombinase expression. This system will be used to manipulate molecular and cellular events, but also to induce an early stage of tumor formation by depleting tumor suppressor genes (e.g. APC in the intestine).

Moreover, the introduction of novel gene(s) will help us in further characterizing the role of expressing cells. E.g. the introduction of fluorescent markers in (putative) different cancer cells pools (e.g. cancer stem cells vs more differentiated tumor cells) allows the isolation of these pools of cells by flow cytometry. Upon isolation, the different pools of cells can be characterized by e.g. gene expression profiling. Moreover, the (combined) introduction of e.g. an exogenous toxin receptor in the same cells allows us to specifically kill these cells upon the administration of the toxin to the mouse, allowing us to determine the consequence of this cell depletion during development and/or in tissue homeostasis. This is also true for over or mis- or overexpression of (mutated) genes, especially oncogenes to induce tumor formation (e.g. the PyMT oncogene in breast tissues).

Administering small molecule compounds/drugs/toxin/chemical or control substances (e.g. inhibitors or agonists of specific pathways), we might be able to rescue or mimic the phenotypes of the in vivo genetic deletions and/or activations and therefore further identify the function of these cells in vivo. If possible and/or relevant, we will always test these small molecule compounds/drugs/chemicals first on in vitro growing organoids and in case relevant effects are observed shift to in the in vivo models.

In a small number of animals (<3%), injuries will be used to manipulate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue. For instance in the past we studied how the retrieval of biopsies induces an injury that recruits immune cells that subsequently release chemokines that induce metastasis.

III) Transplantation tissues/cells/organoids (3.4.4.3 and 3.4.4.4):
To identify the role of various cell types on developmental and cancer processes, we need to transplant (genetically modified) human and murine tissues, cell or organoids in mice. For example, in the past we have transplanted colorectal organoids (3D cultures) in mice to induce colorectal tumor growth. Moreover, we have transplanted red-labelled erythrocytes to long-term label blood vessels in order prevent a more discomfort causing procedure of daily injections of fluorescent-dextran enabling only short-term labeling of blood vessels.

IV) In some cases the transplantation should be combined with the cell type specific gene (in)activation and (over)/(mis)expression) (3.4.4.5 and 3.4.4.6):

The cumulative level of discomfort is dependent on both the intervention (moderate) and the read-out. Therefore for both the ex vivo (3.4.4.5) and intravital imaging (3.4.4.6), the cumulative discomfort is moderate.

Readouts/endpoint
We have three types of readouts:
1) Ex vivo analysis (appendices 3.4.4.1, 3.4.4.3 and 3.4.4.5),
2) Acute Intravital imaging (appendices 3.4.4.2, 3.4.4.4, and 3.4.4.6)
3) Chronic imaging (appendices 3.4.4.2, 3.4.4.4, and 3.4.4.6).

To study the molecular and/or cellular mechanisms of cancer processes, we need to analyze histologically and molecularly the tissues and cells from the mice ex vivo, and established cells lines and organoids (3D culture system) to perform in vitro studies. These cell lines and organoids will enable us to perform some of the studies in vitro. We might also use these cell lines and organoids to generate new tumors upon transplantations.

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments two different strategies will be used: (a) Imaging in an acute experiment under anaesthesia and (b) Implanting an imaging window (such as the breast, skin, abdomen and skull) and repeatedly image through that window. For long-term studies (>24hrs) the animal needs to be imaged by multiple imaging sessions, and therefore imaging windows are required. For some research questions, the tissue needs to be visualized frequently for a relative short-period of time (max 3 days). It is not an option to anesthetize the animal multiple times a day, so the animal will be constantly anesthetized. In this case, an imaging window is not required and an acute imaging experiment will be chosen. We have a lot of experience in the lab with these procedures, and only well trained researchers will perform these experiments.

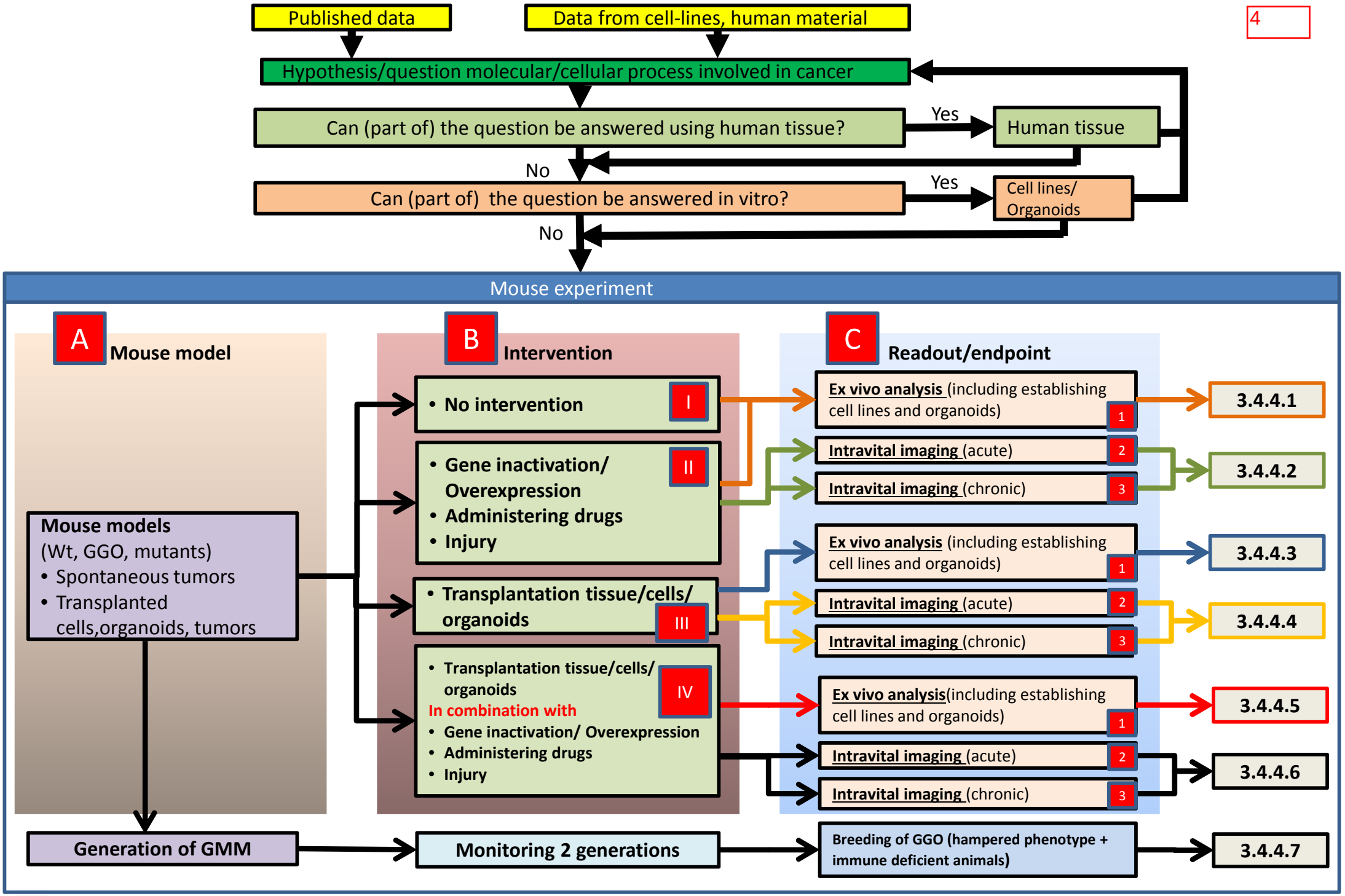
3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

Whenever possible, we will perform pilot studies with the minimum amount of animals possible. We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene/s. For every experiment, we design the experiment with clear go-no-go decisions, to reduce the amount of cumulative discomfort and/or the number of animals. For every experiment, the best trade-off will be made. For example, for most experiments we first consider ex vivo experiments (mild discomfort), before we consider intravital imaging experiments (moderate discomfort). In some experiments we first consider intravital imaging, since either some questions can only be answered by imaging the same tissue over multiple imaging sessions, or it significantly reduces the number of required mice (can be up to a reduction of 20x); multiple time points can be measured in one individual, and there is no inter-mice variation. After completion of the intravital imaging experiments, cells, tissues and organs will be isolated and analyzed to reduce the number of mice required for 3.4.4.1, 3.4.4.3, and 3.4.4.5.

Where possible, mice with inducible alleles will be used, so mice will not display a phenotype before the induction of the alleles. Experiments will be done sequential. When mice show signs of discomfort (e.g. appearance of a tumor), the mouse will be sacrificed and not used for breeding anymore. If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Gene (in) activation interventions (ex vivo analysis)
2	Gene (in) activation interventions (in vivo imaging)
3	Transplantation (ex vivo analysis)
4	Transplantation (in vivo imaging)
5	Transplantation, gene (in) activation interventions (ex vivo analysis)
6	Transplantation, gene (in) activation interventions (in vivo imaging)
7	Generation, welfare assessment and breeding GMM
8	
9	
10	



Indicates choice (A-B), type intervention (I to IV) ,and read-out (1 to 3) as decribed in the project proposal 3.4.2.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.1	Ex-vivo analysis in mouse models and after gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene (transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/specific question (normal tissue development, tumor development, establishing cell

lines/organoids or a cell type specific expression of a fluorescent marker for an immunohistochemical /cell sorting/lineage study)

- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by histology and to isolate the fluorescent expressing cells via FACS sorting, which allows us to analyse (gene expression profile) and culture these cells.
The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g., Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. No Intervention:

I. No experimental interventions:

To visualize for example how stem cells behave during tissue development, we analyze in ex vivo experiments reporter mice in which e.g. stem cells are labelled with GFP.

Interventions:

II Experimental interventions II:

-To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
-The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo.The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
-Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Readout parameter

1. Ex vivo analysis

In all experiments, animals will be killed and embryos, neonatal and/or adult organs will be isolated for detailed analysis of the consequences of the genetic alteration and/or treatment on the (developing) tissue(s). Analysis will include among others histological sections labelled with antibodies or antisense RNA probes, RNA expression analysis, DNA or protein extracts. Also, cells from organs might be isolated

by FACS and/or cultured in vitro (organoids).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 3 time)
   c) intraperitoneal(max. 7 times)
   d) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia
   e) oral (max. 10 times)
3. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   f) oral (max. 10 times)
4. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (max 3 times)
   b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   c) intravenous (max 3 times)
5. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anaesthesia and analgesia
6. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis
   a) Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.
   b) Embryo's and neonates (until P5): will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen (or the brains dissected and fixed).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Embryos(> E13): max. 200
Neonates(until weaning): max. 100

Adult: max. 4000
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
We have estimated the number of animals based on experience over the past 5 years. To give an idea of typical experiments we have done in the past 5 years:

1) Animals (including embryos, neonates and adults) are sacrificed at various developmental stages without experimental intervention. To examine e.g. the histological morphology of a stage, typically 6 mice are required per time point, and typically 5 time-points are taken. For example such a typical experiment requires 30 mice. On average, we had typically 8 of this type of projects per year, so over a 5 year time span we expect to require approximately 40 of these typical experiments.
2) In the case of generating tumors in the intestine via the inactivation of a single gene (e.g. Apc) in the stem cells (via Lgr5-ires-creert) a maximum of 30 mice may be required (5 (number of mice per group) * 6 (different time points required to follow the development of intestinal tumors over time). On average, we had typically 6 of this type of projects per year, so over a 5 year time span we expect to require approximately 30 of these typical experiments.
3) Influence of e.g. drugs on development of intestinal tumors as described above in 2: Two groups of mice as described above in 2 are typically treated with vehicle and the drug. So 60 in total (30 for vehicle, 30 for the drug). On average, we had typically 7 of this type of projects per year, so over a 5 year time span we expect to require approximately 35 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the

molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice need for breeding.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed

Indicate the likely incidence.

Expected 0% within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Embryo's(>E13): mild 100%
Neonates: mild 100%
Adult: mild 96%, moderate 1% <1day, moderate <3% constantly due to implantation pellet/pump

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.2	In vivo imaging in mouse models and after gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine with in vivo imaging the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g. tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g. tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by in vivo imaging and to isolate the fluorescent expressing cells after the experiment via FACS sorting, which allows us to analyze (gene expression profile) and culture these cells.
The introduction of e.g. a toxin receptor (e.g. Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g. Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. <u>No Intervention:</u>

I. No experimental interventions:

To visualize for example how stem cells behave during tissue development, we analyze in ex vivo experiments reporter mice in which e.g. stem cells are labelled with GFP.

<u>Interventions:</u>

II Experimental interventions II:

- To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
- The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo. The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
- Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Readout parameters

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments two different strategies will be used:

1. Imaging in an acute experiment under anesthesia. This is the method of choice for the imaging of processes that need to be imaged frequently (time scale of hours) for a short period of time (max 72 hours). In this case it is not an option to anesthetize the animal

multiple times a day.
2. Chronic imaging. To study processes at a timescale of days chronic imaging is the method of choice.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 1 time)
   c) intraperitoneal(max. 5 times)
   d) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia
   e) oral (max. 10 times)
3. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   f) oral (max. 10 times)
4. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (max 3 times)
   b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   c) intravenous (max 3 times)
5. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anaesthesia and analgesia
6. Intravital imaging. For the imaging experiments one of the two different strategies will be used:
   1) Acute imaging: Surgical exposure of the imaging site under adequate anaesthesia and analgesia. Such an experiment will last up to 72 hours
   2) Chronic imaging: In these situation prior to the imaging period an imaging window is implanted (such as the breast, skin, abdomen and skull) (one window per animal) followed by the repeated imaging sessions under isoflurane anesthesia. From experience we know that for the implantation of windows in the skin does not lead to post-operative discomfort and post-operative analgesia is therefore extensive postoperative analgesia is not indicated. In a number of cases this is contra-indicated for the processes under study.

7) All animals will be killed while still under anesthesia for ex vivo analysis of the isolation of organs or tumors:
   a) perfusion fixation under lethal dose of Nembutal.
   b) via cervical dislocation under isoflurane anaesthesia

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.

Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments.

Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 5000
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
We have estimated the number of animals based on experience over the past 5 years. To give an idea of typical experiments we have done in the past 5 years:

1) Acute imaging without experimental intervention. For example, we used this experiment to see how frequent Lgr5+ stem cells in the intestine divide at various locations within the stem cell niche. We know from experience that we need to image at least 12 mice to quantitatively say whether these cells have migratory potential or not. On average, we had typically 10 of this type of projects per year, so over a 5 year time span we expect to require approximately 50 of these typical experiments.
2) Acute imaging with experimental intervention. For example, we used this experiment to test the migration potential of tumor cells that have "high-jacked" stem cells properties in breast tumors, mice will be imaged acutely that overexpress PyMT oncogene in the mammary gland. We know from experience that we need to image at least 6 mice to quantitatively say whether these cells have migratory potential or not. On average, we had typically 20 of this type of projects per year, so over a 5 year time span we expect to require approximately 100 of these typical experiments.
3) Chronic imaging without experimental intervention. For example, we used this experiment to see how the progeny of Lgr5+ stem cell outcompetes the progeny of another Lgr5+ stem cell. To study this, we typically image the progeny over multiple days. We typically image at least 20 mice to quantitatively say something about competition. On average, we had typically 20 of this type of projects per year, so over a 5 year time span we expect to require approximately 100 of these typical experiments.
4) Chronic imaging with experimental intervention. For example, we used this experiment to see how the progeny of Lgr5+ stem cell in which e.g. the APC is depleted outcompetes the progeny of a wild-type Lgr5+ stem cell. To study this, we typically image the progeny over multiple days. We typically image at least 20 mice to quantitatively say something about competition. On average, we had typically 18 of this type of projects per year, so over a 5 year time span we expect to require approximately 90 of these typical experiments.

For the chronic in vivo imaging experiments in some cases the animals can no longer be used for the experiments (tumour at a location not suitable for imaging, problems with the window. This happens in <10% of the cases.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice needed for breeding.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier than the humane endpoints.
It is expected that no animals (0%) will be experiencing more than mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible

Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs. From prior experience we know that window loss is not a source for additional discomfort.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.
It is unclear why some animals may lose their windows.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
Loss of window
Growth of tumor: In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Mild discomfort : interventions resulting in mild discomfort in combination with acute imaging (15%)
Moderate discomfort: interventions resulting in moderate discomfort in combination with acute imaging (15%)
Moderate discomfort : Interventions in combination with chronic imaging (70%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed while still under anesthesia for imaging to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.3	Ex-vivo analysis in mouse models and after transplantation tissues/cells/organoids

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the flowchart in appendix 1.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker for an

immunohistochemical study)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

B. Intervention III:

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to localize these cells by histology and to isolate the fluorescent expressing cells via FACS sorting, which allows us to analyze (gene expression profile) and culture these cells. In some cases, mice get a special diet (but this does not lead to any discomfort).

C. 1. Ex vivo analysis

In all experiments, animals will be killed adult organs will be isolated for detailed analysis of the consequences of transplantation of tissue/cells/organoids. Analysis will include among others histological sections labelled with antibodies or antisense RNA probes, RNA expression analysis, DNA or protein extracts. Also, cells from organs might be isolated by FACS and/or cultured in vitro (organoids).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:

a) in diet or drinking water (max. 1 time, < 2 wks)
b) subcutaneous (max. 1 time)
c) intraperitoneal(max. 5 times)
d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
f) oral (max. 10 times)

4. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:

a) intraperitoneal (max 3 times)
b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
c) intravenous (max 3 times)

5. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis

Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 4500
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize tumor cells that have "high-jacked" stem cell properties in colorectal tumors, these cells need to be isolated by flow cytometry to subsequently analyze them by RNAseq. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC is depleted in the stem cells (via lgr5-GFP ires-creert). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. Based on the expression of GFP, these cells can then be isolated by flow cytometry. For such a typical experiment, a maximum of 30 mice may be required (15 donor mice and 15 recipient mice per group). For some experiments, multiple time points are required. On average, we had typically 30 of this type of experiments per year, so over a 5 year time span we expect to require approximately 150 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of gene-inducing agents animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day.

Explain why these effects may emerge.

Tumordevelopment and/or inflicted tissue damage due to genetic alterations

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed

Indicate the likely incidence.

Expected 0% within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Adult: mild 96%, moderate 1% <1day, moderate <3% constantly due to implantation pellet/pump

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.4	In vivo imaging in mouse models and after transplantation tissues/cells/organoids

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.

Our aim is to determine with [redacted] the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell

lines/organoids or a cell type specific expression of a fluorescent marker)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

B. Intervention III:

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to visualize these cells by intravital imaging. In some cases, mice get a special diet (but this does not lead to any discomfort).

C. Readout parameters

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments two different strategies will be used:

2. Imaging in an acute experiment under anaesthesia. This is the method of choice for the imaging of processes that need to be imaged frequently (time scale of hours) for a short period of time (max 72 hours). In this case it is not an option to anesthetize the animal multiple times a day.

Chronic imaging. To study processes at a timescale of days chronic imaging is the method of choice.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Intravital imaging either:
   - Acute imaging: Surgical exposure of the imaging site under adequate anaesthesia and analgesia. Such an experiment will last up to 72 hours
   - Chronic imaging: In these situation prior to the imaging period an imaging window is implanted (such as the breast, skin, abdomen and skull) (one window per animal) followed by the repeated imaging sessions under isoflurane anesthesia. From experience we know that for the implantation of intracutaneous windows does not lead to post-operative discomfort and post-operative analgesia is therefore extensive postoperative analgesia is not indicated. In a number of cases this is contraindicated for the processes under study.
4. All animals will be killed while still under anesthesia for ex vivo analysis of the isolation of organs or tumors:
   a) perfusion fixation under lethal dose of Nembutal.
   b) via cervical dislocation under isoflurane anesthesia

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments.
Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 2000
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize whether the tumor cells that have "high-jacked" stem cell properties have also acquired migratory properties, the motility of these cells need to be visualized by intravital microscopy. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC is depleted in the stem cells (via lgr5-GFP ires-creert). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. These cells can then be visualized by acute intravital imaging to visualize e.g. cell migration and chronic intravital imaging to visualize e.g. the formation of progeny. For such a typical experiment, a maximum of 20 mice may be required (10 donor mice and 10 recipient mice per group).

1) Acute imaging: On average, we had typically 4 of this type of projects per year, so over a 5 year time span we expect to require approximately 20 of these typical experiments.
2) Chronic imaging: On average, we had typically 16 of this type of projects per year, so over a 5 year time span we expect to require approximately 80 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth

and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken

to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies aremuch earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.

Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs. From prior experience we know that window loss is not a source for additional discomfort.

Explain why these effects may emerge.

Tumordevelopment and/or inflicted tissue damage due to transplantation of tissues/cells/organoids.
It is unclear why some animals may lose their windows.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
Loss of window
Growth of tumor: In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Mild discomfort : mild discomfort due to intervention, acute imaging (12%)
Moderate discomfort: moderate discomfort due to intervention, acute imaging (8%)
Moderate discomfort : All chronic imaging experiments (80%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed while still under anesthesia for imaging to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.5	Ex-vivo analysis in mouse models and after transplantation tissues/cells/organoids, Gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker for an immunohistochemical study)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze (GM) mice in which cells/tissues/organoids are transplanted and the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In particular, when Cre is expressed in mice harboring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell) of the mouse depending on the specificity and timing of recombinase expression. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by histology and to isolate the fluorescent expressing cells via FACS sorting, which allows us to analyze (gene expression profile) and culture these cells.
The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g., Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. <u>Interventions IV:</u>

- To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to visualize these cells by intravital imaging.To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
- The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo.The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
- Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Ex vivo analysis

In all experiments, animals will be killed adult organs will be isolated for detailed analysis of the consequences of transplantation of tissue/cells/organoids. Analysis will include among others histological sections labelled with antibodies or antisense RNA probes, RNA expression analysis, DNA or protein extracts. Also, cells from organs might be isolated by FACS and/or cultured in vitro (organoids).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 3 time)
   c) intraperitoneal(max. 7 times)
   d) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia
   e) oral (max. 10 times)
4. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   f) oral (max. 10 times)
5. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (max 3 times)
   b) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   c) intravenous (max 3 times)
6. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anesthesia and analgesia
7. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis
   a) Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.
   b) Embryo's and neonates: will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen (or the brains dissected and fixed).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 1650
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize how inactivation of a gene (e.g. Kras) affects tumor cells that have "high-jacked" stem cell properties in colorectal tumors, these cells need to be isolated by flow cytometry to subsequently analyze them by RNAseq. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC and Kras are depleted in the stem cells (via lgr5-GFP ires-CreERTt). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. Based on the expression of GFP, these cells can then be isolated by flow cytometry. For such a typical experiment, a maximum of 60 mice may be required (15 donor mice and 15 recipient mice per group. We need two groups: one control group in which Kras is not depleted and one group in which Kras is depleted). On average, we had typically 5 to 6 of this type of projects per year, so over a 5 year time span we expect to require approximately 27,5 of these typical experiments.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used as dormicum and Temgesic to releave pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed

Indicate the likely incidence.

Expected 0% within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Adult: mild 96%, moderate 1% <1day, moderate <3% constantly due to implantation pellet/pump

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.
☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.6	In vivo imaging in mouse models and after transplantation tissues/cells/organoids, Gene inactivation/overexpression, administering drugs and injury

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart.
Our aim is to determine in ex vivo analysis the molecular/cellular mechanisms required for tissue homeostasis/development, tumor initiation, tumor progression, cancer diagnosis, cancer prevention, and cancer treatment via the analysis of (compound) mouse models.

The different components of the proposed experiments are (see also the flowchart in appendix 1):

A. <u>Mouse models:</u>

There are several considerations to choose for a specific animal model:

- Wt mice,
- Genetically modified mice that expressed a fluorescent marker in a specific cell type/lineage. In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to induce the expression of the fluorescent marker.
- Genetically modified mice that changes expression of a functional gene(transgene knockout). In some cases transgene inducing or deleting agents (e.g tamoxifen) are administered to change the expression of the functional gene.
- Spontaneous tumor model
- Immune-deficient/wt recipient mouse

The choice will in all cases based on the combination of the following considerations:

- Aim/ specific question (normal development, tumor development, establishing cell lines/organoids or a cell type specific expression of a fluorescent marker for an immunohistochemical study)
- Type of tumor
- Aim/readout parameters (e.g. static vs dynamic, long-term vs short-term dynamics)

To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze (GM) mice in which cells/tissues/organoids are transplanted and the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. In some cases, mice get a special diet (but this does not lead to any discomfort).
We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In particular, when Cre is expressed in mice harboring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell) of the mouse depending on the specificity and timing of recombinase expression. In this way the desired gene modification can be restricted to certain developmental stage, organ (e.g. intestine), cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes.
The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by in vivo imaging.
The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g., Diphtheria toxin). This study allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.
If the required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (3.4.4.7).

B. <u>Interventions IV:</u>

- To identify/validate for example which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze mice in which tissues/cells/organoids are (mostly orthotopically) transplanted. The presence of e.g. a fluorescent marker in transplanted tissues/cells/organoids allows us to visualize these cells by intravital imaging. To identify/validate which molecular and cellular processes are important for development and/or tissue homeostasis, for misregulating the processes leading to tumor initiation, the growth and metastasis of tumors, and the development of therapy resistance, we will analyze GM mice in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed.
- The administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells in vivo. The mice might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stems and their derivatives.
- Injuries (such as the surgical removal of a tumor, taking biopsies (not for the analysis, but for the injury), making a wound) will be made to investigate for example the recruitment of cell types (e.g. immune cells) with subsequent effects on expression profile of different cells in the tissue and the tumor.

C. Readout parameters

To study dynamic processes that are missed in static histological images, in vivo imaging will be performed. For the imaging experiments one of the two different strategies will be used:

1. Imaging in an acute experiment under anesthesia. This is the method of choice for the imaging of processes that need to be imaged frequently (time scale of hours) for a short period of time (max 72 hours). In this case it is not an option to anesthetize the animal multiple times a day.
2. Chronic imaging. To study processes at a timescale of days chronic imaging is the method of choice.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

1. Tissue sampling for genotyping and identification via ear and tail biopsy resp. under anaesthesia (4% (isoflurane/oxygen).
2. Transplantation of tissues/cells/organoids under adequate anaesthesia and analgesia
3. Administration of transgene inducing or deleting agents or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. 1 time, < 2 wks)
   b) subcutaneous (max. 3 time)
   c) intraperitoneal(max. 7 times)
   d) implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   f) oral (max. 10 times)
4. Administration of small molecule compounds, drugs, toxin, chemicals or control substances alone or in combination, continuously or intermittently by one or more of the following routes:
   a) in diet or drinking water (max. time, <2 wks)
   b) subcutaneous (max. 10 time)
   c) intraperitoneal (max. 10 times)
   d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (1 time)
   e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (1 time)
   f) oral (max. 10 times)
5. (Optional) Administration of a labelling agent (e.g. BrdU) via one of the following routes:
   a) intraperitoneal (1 time)
   b) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (1 time)
   c) intravenous (1 time)
6. (Optional) To mimic the injury resulting from e.g. surgical removal of a tumor or obtaining a biopsy, (tumor) tissues will be 'injured' under adequate anaesthesia and analgesia
7. Intravital imaging either

   - Acute imaging: Surgical exposure of the imaging site under adequate anaesthesia and analgesia. Such an experiment will last up to 72 hours
   - Chronic imaging: In these situation prior to the imaging period an imaging window is implanted (such as the breast, skin, abdomen and skull) (one window per animal) followed by the repeated imaging sessions under isoflurane anesthesia. From experience we know that for the implantation of intracutaneous windows does not lead to post-operative discomfort and post-operative analgesia is therefore extensive postoperative analgesia is not indicated. In a number of cases this is contraindicated for the processes under study.
8. All animals will be killed and organ(s)/tissue will be isolated for ex vivo analysis
   a) Adult mice: via CO2/O2 method or perfusion fixation under lethal dose of Nembutal.
   b) Embryo's and neonates: will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen (or the brains dissected and fixed).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (Wt, genetically modified, mutants)
Origin: Hubrecht institute/external licensed breeders.
Adult: max. 2800
All animal studies in this project will exclusively involve mice. Up to today, there are no alternative methods to fully understand tissue development/homeostasis and cancer in the context of the whole organism. For the majority of the proposed studies, the mouse is the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

For the type of experiments described in this procedure, it is difficult to calculate the exact number of animals, since for various experimental setups different amount of mice are required, and the variation and outcome of the experiments are unknown. The number of mice required depends on the mouse model (e.g tumor incidence, tumor heterogeneity), the type of intervention and the characteristics of the measuring parameters and can therefore only be described in global and strategic terms. For these reasons we have provided a total number of mice based on experience over the past 5 years with these types of experiments in our group. Before we will start an experiment we will write an application to the IVD. In this application we will exactly describe which considerations, facts and results have led to the proposition of the number of animals needed for these experiments.
To give an idea of a typical experiment in this appendix: E.g. if we want to characterize how inactivation of a gene (e.g. Kras) affects the migration properties of tumor cells that have "high-jacked" stem cell properties, the motility of these cells need to be visualized by intravital microscopy. For example, for this experiment we want to isolate pieces of colon from mice in which the tumor suppressor gene APC and Kras are depleted in the stem cells (via lgr5-GFP ires-creert). These mice develop tumors throughout the intestine, and reach human end point, due to dysfunctional intestine, before tumors progress to a stage in which cells acquire stem cell properties. Therefore, pieces of colon are transplanted in recipient mice to initiate the growth of a single tumor. Since just one tumor is formed upon transplantation, the intestine is functional and the tumor can progress to a stage where cells adapt stem cell properties and start to express GFP by the Lgr5 stem cell promotor. Based on the expression of GFP, stem cells can be visualized by acute intravital imaging to visualize e.g. cell migration and chronic intravital imaging to visualize e.g. the formation of progeny. For such a typical experiment, a maximum of 40 mice may be required (10 donor mice and 10 recipient mice per group. We need two groups: one control group in which Kras is not depleted and one group in which Kras is depleted).

1) Acute imaging: On average, we had typically 9 of this type of projects per year, so over a 5 year time span we expect to require approximately 45 of these typical experiments.
2) Chronic imaging: On average, we had typically 5 of this type of projects per year, so over a 5 year time span we expect to require approximately 25 of these typical experiments.

For the chronic in vivo imaging experiments in some cases the animals can no longer be used for the experiments (tumour at a location not suitable for imaging, problems with the window. This happens in 10%< of the cases.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☐ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the import or generation and subsequently analysis of (compound) GGM to study cancer models, we first will extensively analyze cell lines, existing tissue patient material and/or organoids. However, animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive tissue homeostasis/development, cancer initiation, growth and metastasis.

We make extensive use of human material and in vitro experiments using cell lines and organoid culture (3D cultures) where possible, which extensively reduces the animal numbers. The use human material and in vitro cultures allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum.

Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.

Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps..

If homozygous mice doesn't show any discomfort we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances animals will be experiencing no follow up effects.
Animals bearing tumors will never reach end-stage clinical effects. The scientific endpoints of all studies are much earlier then the humane endpoints.
It is expected that no animals (0%) will be experiencing more then mild discomfort due to the genetic modification. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analyzed when possible.

Animals may lose their window (<5%). In the vast majority of these animals (>95%), we notice signs of detachment of the window far before it becomes lose, and the mice will be sacrificed. In the remaining animals, the window loss will be noticed within 24hrs. From prior experience we know that window loss is not a source for additional discomfort.

Explain why these effects may emerge.

Tumor development and/or inflicted tissue damage due to genetic alterations and/or administration of small molecule compounds/drugs/chemicals/toxins.

It is unclear why some animals may lose their windows.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort.
Loss of window
Growth of tumor: In all cases the guidelines of the Code of Practice Animals in Cancer research will strictly be followed.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Moderate discomfort: 100%

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80102
1.2	Provide the name of the licenced establishment.	Koninklijke Nederlandse Academie van Wetenschappen (Hubrecht Institute)

1.3 List the serial number and type of animal procedure.

Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.

Serial number	Type of animal procedure
3.4.4.7	Generation, welfare assessment and breeding of GMM/mutants with hampered phenotype

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Creation of genetically mice via DNA/RNA injection into oocyte, injection of genetically modified ES cells into blastocysts and/or via the CRISPR/Cas9 system.

Welfare assessment according to the Consensus document on genetically altered animals . Newcompound mouse models and new created transgenic lines and/or KO lines generated via classical methods and/or novel combinations of these aforementioned lines will be monitored for 2 generations to determine the absence or presence a phenotype with constitutional discomfort.

For some transplantation experiments (e.g. when human organoids are transplanted), immune deficient acceptor mice are required. We breed our own immune deficient NOD-SCID mice. For this, we have 6-10 breeding pairs that will be replaced every 6 month that leads to offspring that will be used for experiments. Since these mice are housed under proper barrier conditions, they do not have a hampered phenotype. According to the Consensus document on genetically altered animals, breeding with these animals is considered an animal experiment and should therefore be part of the license.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Generation of new lines:
1) Superovulation.
a) Administration of gonadotropin's (2 times) by subcutaneous or intraperitoneal injections followed by mating.
b) Animals will be killed for the isolation of early embryos.

2) Embryo recipients.
a) Recipients for embryo transfer will be rendered pseudo-pregnant by mating with a sterile (vasectomized) male.
b) Genetically modified embryos will be implanted surgically or non-surgically into the reproductive tract.
c) Embryo recipients, not as part of an experiment, will be killed after weaning of the pups at three weeks of age.

3) Weaned pups at 3 weeks of age: Tissue sampling for genotyping and/or identification via tail and ear cut, respectively, under anesthesia (isoflurane).

Animals are killed by O2/CO2 method.

Breeding immune deficient acceptor mice.

Welfare assessment:
We daily check the mice on several parameters (overall appearance, size, confirmation and growth, coat condition, behavior, clinical signs, relative size and numbers) as has been described in the Directive 2010/63/EU: corrigendum of 24 Jan. 2013.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Statistical analysis doesn't play a role for these types of experiments. We will use state of the art techniques. All techniques are proven to be effective in generating GM mice with a minimum number of mice possible.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus: genetically modified and wild type adult mice. All vasectomized males which will be obtained from a registered commercial company, all other mice are obtained from our own Institute, an establishment licensed breeder by the NVWA, or from a registered commercial company.

Generation of GG mice: we expect to generate max. 10 new lines over the next 5 years. For the creation of a new GM mouse line we will use on average max. 150 mice (according to the besluit biotechnologie). Therefore in total max. 1.500 mice.

Welfare assessment: we expect to generate over the next 5 years 10 new (compound) GM lines for which we have to perform the welfare assessment. For 2 generations, 7 males and 7 females control and GM mice. We therefore need in total: 10 (new (compound) lines) * 2 (generation) * 28 ((7 male +7 female = 14 GM mice) + (7 male + 7 female = 14 control mice))= 560 mice.

Therefore in total max. 1.500 (generation of GGM) + 560 (welfare assessment) = 2060 mice
Of note
The majority of the newly generated GG mice will be floxed mice which are not part of the welfare assessment protocol.
We will not breed mice showing a hampered phenotype and but instead will sacrifice them.

For some transplantation experiments (e.g. when human organoids are transplanted), immune

deficient acceptor mice are required. We breed our own immune deficient mice. For this, we have 6-10 breeding pairs that will be replaced every 6 month that leads to offspring that will be used for experiments. So the max number of animals will be: 10 (pairs) x 2 (male and female) x 2 (twice a year) x 5 (for 5 years) = 200 animals for breeding.

These 10 breeding pairs generate on average 28 pups per week that will be used in appendix 3.4.4.3, 3.4.4.4, 3.4.4.5 and 3.4.4.6. These animals are described and counted in these appendixes.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before we consider the generation of a new (compound) GM Mice we first will extensively analyze cell lines, existing tissue patient material and/or organoids. Only the in vitro experiments do not provide sufficient information or does not address completely the research question/hypothesis, we will consider the generation of a novel GM mice.
Animal studies are unavoidable if we seek comprehensive knowledge and understanding of molecular and cellular mechanisms of tissue homeostasis and cancer.
The CRISPR/Cas9 system allows us, if required, to genetically modify up to 5 different genes at the same time. This strongly reduce the number of mice used for the generation and/or breeding of these compound mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, isoflurane will be used general anaesthesia and Temgesic to relieve pain.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

We don´t expect to find other adverse effect. This is the direct result of how we create our constructs for the generation of GM mice

Explain why these effects may emerge.

We don´t expect to find other adverse effect

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☒ No > Continue with question K.

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Donors: moderate 100%
Fosters: moderate 100%
GM mice: no 99%
GM mice: mild 1%
Immune deficient mice: no 100%
Monitoring mice: no 100%

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals are killed to use their organs in ex vivo analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

AVD-801002015125 Overzicht aantal muizen, groepen en ongerief

Totaal aantallen: embryo's 200, pasgeboren 100 en volwassen 22.210
Muizen: 52% mild ongerief en 48% matig ongerief

	Procedure	Group	Animals	mild	moderate	Total group
3.4.4.1	Gene (in) activation interventions (ex vivo analysis)	Group 1a	Mice embryo's (>E13)	200		
		Group 1b	Mice neonates (until weaning)	100		
		Group 1c	Mice adults 99%	3840		
		Group 1d	Mice adults 4%		160	4300
3.4.4.2	Gene (in) activation interventions (in vivo imaging)	Group 2a	Mice adults 15%	750		
		Group 2b	Mice adults 85%		4250	5000
3.4.4.3	Transplantation (ex vivo analysis)	Group 3a	Mice adults 96%	4320		
		Group 3b	Mice adults 4%		180	4500
3.4.4.4	Transplantation (in vivo imaging)	Group 4a	Mice adults 12%	240		
		Group 4b	Mice adults 88%		1760	2000
3.4.4.5	Transplantation Gene (in) activation interventions (ex vivo analysis)	Group 5a	Mice adults 96%	1584		
		Group 5b	Mice adults 4%		66	1650
3.4.4.6	Transplantation Gene (in) activation interventions (in vivo imaging)	Group 6	Mice adults 20%		2800	2800
3.4.4.7	Generation Welfare assessment and Breeding of GMM	Group 7a	Mice adults 100% (generation GMM)		1500	
		Group 7b	Mice adults (welfare assessment)	554		
		Group 7c	Mice adults (welfare assessment)		6	
		Group 7d	Immune deficient mice adults (breeding)	200		2260

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Aanvraagnummer
AVD801002015125

Bijlagen
2

Datum 23-06-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 22 juni 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD801002015125. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. Zodra uw aanvraag compleet is, ontvangt u binnen veertig werkdagen een beslissing op uw aanvraag. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan wordt uw aanvraag buiten behandeling gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	80100
Naam instelling of organisatie:	Kon. Ned. Academie van Wetenschappen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	54667089
Postbus:	19121
Postcode en plaats:	1000 GC AMSTERDAM
Tenaamstelling van het rekeningnummer:	Hubrecht Instituut/Nederlandshersen Instituur

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	Group Leader
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 juni 2015
Geplande einddatum:	1 juni 2020
Titel project:	The molecular and cellular mechanisms of tumor initiation, growth, metastasis and thera
Titel niet-technische samenvatting:	Het begrijpen van het ontstaan en ontwikkeling van kanker, uitzaiingen en resistentie
Naam DEC:	DEC-KNAW
Postadres DEC:	[redacted] Amsterdam
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 741,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Amsterdam
Datum:	1 juni 2015

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen
[redacted]
Postbus 19121
1000 GC AMSTERDAM

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
www.zbo-ccd.nl
0900 28 000 28 (10 ct/min)

Onze referentie
Aanvraagnummer
AVD801002015125

Bijlagen
2

Datum 23-06-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 23 juni 2015
Vervaldatum: 23 juli 2015
Factuurnummer: 201570125

Omschrijving	**Bedrag**
Betaling leges projectvegrunning dierproeven Betreft aanvraag AVD801002015125	€ 741,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

11 januari 2015

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de bijbehorende toelichting, waarin elke stap in het beoordelingsproces wordt toegelicht

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD/801002015125
2. Titel van het project: The molecular and cellular mechanisms of tumor initiation, growth, metastasis and therapy resistance.
3. Titel van de NTS: Het begrijpen van het ontstaan en ontwikkeling van kanker, uitzaaiingen en resistentie tegen therapie.
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
   - wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 13-05-2015
   - ✓ aanvraag compleet: 15-06-2015
   - ✓ in vergadering besproken: 21-05-2015
   - ✓ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en): n.v.t.
     besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen:
     aanpassing aanvraag:
   - ✓ advies aan CCD: 22-06-2015
7. Eventueel horen van aanvrager
   - Datum: n.v.t.
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
8. Correspondentie met de aanvrager:
   - Datum 27-05-2015
   - Strekking: completering van de aanvraag
   - Datum antwoord: 15-06-2015
   - Strekking van de antwoorden: de aanvraag is gecompleteerd
9. Eventuele adviezen door experts (niet lid van de DEC): geen

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig. Het omvat dierproeven in de zin der wet.

2. De aanvraag betreft een nieuwe aanvraag. Er is enige overlap met een aantal al van een positief advies voorziene DEC-protocollen.
3. De DEC is competent om over deze projectvergunningsaanvraag te adviseren. De benodigde expertise op dit wetenschappelijk terrein is aanwezig binnen de DEC. Geen van de DEC-leden is betrokken bij het betreffende project.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering: n.v.t.

C. Beoordeling (inhoud):

1. Het project is wetenschappelijk verantwoord.

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

3. De doelstelling, in relatie tot de uitvoering, is helder omschreven; te weten het verkrijgen van fundamenteel wetenschappelijke inzichten in 1) de biologische mechanismen van processen die belangrijk zijn voor het ontstaan, groei, uitzaaiing en therapieresistentie van tumoren, en 2) het ontwikkelen/verbeteren van kankertherapieën. Op termijn kunnen de resultaten leiden tot nieuwe behandelingsmethoden voor patiënten met kanker.

   Het project richt zich op tumorvorming, het ontstaan van uitzaaiingen en therapieresistentie. Binnen dit project zijn de belangrijkste doelen het begrijpen wat de rol is van stamcellen en kankerstamcellen en wat de betrokkenheid is van de micro- en macro-omgeving waarin deze cellen zich bevinden. Het fundamenteel wetenschappelijke belang acht de DEC substantieel.

   Het verkrijgen van deze fundamenteel wetenschappelijke kennis is essentieel voor het ontwikkelen van nieuwe en/of verbeterde therapeutische strategieën voor de behandeling van kanker en dit is naar de mening van de DEC een substantieel belang. Het project dient hiermee een belangrijk maatschappelijk belang, gezien de grote groep patiënten met tumoren.

4. De gekozen strategie en experimentele aanpak in combinatie met de infrastructuur op het [redacted] en de expertise van de betrokken onderzoeksgroep bieden een realistisch uitzicht op het behalen van de beoogde doelstellingen binnen gevraagde looptijd van het project. Het project bouwt voort op een langlopende lijn van onderzoek van een grote groep onderzoekers. Over de afgelopen jaren zijn met een vergelijkbare strategie en aanpak belangrijke wetenschappelijk resultaten behaald, resulterend in vele publicaties in vooraanstaande tijdschriften. Het onderzoek wordt financieel gesteund door verschillende onafhankelijke subsidiegevers. Er zijn internationale samenwerkingsverbanden met andere laboratoria actief in dit onderzoeksveld.

Verder zijn er nauwe banden met de kliniek waardoor er een sterke wisselwerking ontstaat tussen klinisch relevante vragen en het beschreven fundamentele onderzoek waar de dierproeven een deel van uit maken.

5. Alle dieren worden gefokt voor het gebruik in dierproeven, er is geen sprake van afwijkende huisvesting en/of hergebruik. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit het wild. De toegepaste methoden voor anesthesie/euthanasie zijn conform de Richtlijn.

6. Het cumulatieve ongerief gepaard gaand met de dierproeven, zoals beschreven in de zeven verschillende type dierproeven, is naar inschatting van de DEC licht (Type dierproef 1, 3, en 5) of matig (Type dierproef 2, 4, 6 en 7). Er een beperkt risico op onbedoelde bijwerkingen. Deze inschatting van de DEC is in overeenstemming met het niveau van cumulatief ongerief zoals dat is geclassificeerd door de onderzoekers. Dit is gebaseerd op hun ruime ervaring met de gebruikte modellen in vergelijkbare dierproeven.

7. Binnen het project wordt maximaal gebruik gemaakt van methoden die de voorgestelde dierproeven geheel of gedeeltelijk **vervangen.** Een belangrijk onderdeel van de experimentele strategie is de gefaseerde opzet. In de eerste fase, voorafgaand aan de dierproeven, vindt een uitgebreid onderzoek plaats met weefsel afkomstig van patiënten en cellijnen. Na deze fase zijn er go/no-go-beslissingsmomenten, voordat tot het uitvoeren van dierproeven wordt besloten. Nieuwe inzichten in de processen die de initiatie, groei en metastase van tumorcellen reguleren kunnen op dit moment alleen maar verkregen worden in een intact organisme. Deze processen, waarbij verschillende typen cellen betrokken zijn binnen een gecompliceerde anatomische context, zijn zeer complex en kunnen niet met cellijnen worden bestudeerd. Naar het oordeel van de DEC zijn er geen alternatieven beschikbaar voor het voorgestelde gebruik van intacte dieren om te doelstelling van dit project te realiseren.

8. In het project wordt optimaal tegemoet gekomen aan de vereisten van **vermindering** van dierproeven. De onderzoeksgroep heeft een jarenlange ervaring opgebouwd met dit soort experimenten en door een veelal gefaseerde opzet wordt per experiment niet meer dan het minimaal benodigde aantal dieren ingezet. Voorafgaand aan de kwantitatieve experimenten wordt op basis van literatuurgegevens, eigen historische data of een specifiek hiertoe uitgevoerd pilot experiment de groepsgrootte bepaald. Technieken en procedures worden zorgvuldig toegepast. Het aantal te gebruiken dieren is realistisch geschat.

9. De uitvoering van het project is in overeenstemming met de vereisten van **verfijning** van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.
Bij de opzet wordt rekening gehouden met dierenwelzijn en wel op de volgende manieren: 1) het gebruik van adequate anesthesie en analgesie waar nodig, 2)

een intensieve monitoring van de proefdieren na de inductie van tumoren, 3) het gebruik van weefselspecifiek genetisch-gemodificeerde muizen, 4) een monitoring op het optreden van onverwacht constitutioneel ongerief van nieuwe gecreëerde genotypes.
Er is geen sprake van belangwekkende milieueffecten.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en is geformuleerd in begrijpelijke taal. De NTS voldoet daarmee aan de eisen zoals gesteld in artikel 10.a.1.7 van de Wod.

D. Ethische afweging

De centrale vraag voor de ethische afweging is of het belang van het doel van dit project opweegt tegen het ongerief dat de dieren ondergaan (geclassificeerd als licht of matig). Het doel van het project is het verkrijgen van fundamenteel wetenschappelijke inzichten in: 1) de biologische mechanismen van processen die belangrijk zijn voor het ontstaan, groei, uitzaaiing en therapieresistentie van tumoren, en 2) het ontwikkelen/verbeteren van kankertherapieën.
Het onderzoek is primair fundamenteel wetenschappelijk van karakter. De verwachting is dat de resultaten op den duur kunnen bijdragen aan nieuwe of verbeterde therapieën voor kankerpatiënten wat voor een grote groep patiënten van groot belang is om te overleven.

Het fundamenteel wetenschappelijke onderzoek in dit project is van aangetoonde en excellente kwaliteit. De onderzoeksgroep beschikt over ruime ervaring met de gekozen onderzoeksstrategie en met de zeven beschreven type dierproeven.

De classificatie van het ongerief van de dieren in de verschillende typen dierproeven is licht of matig. Bij de het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van de vervanging, vermindering en verfijning van de dierproeven. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald.

De DEC is van mening dat de resultaten van dierproeven zullen bijdragen aan het behalen van het geformuleerde doel en schat de kans op het realiseren van de fundamenteel wetenschappelijke doelstellingen in als hoog. Het project is uit wetenschappelijk oogpunt verantwoord. De verkregen fundamenteel wetenschappelijke kennis is essentieel om te kunnen komen tot nieuwe therapeutische benaderingen of van een verbetering van bestaande therapieën in patiënten met tumoren. Het gaat om een grote groep patiënten met uiteenlopende, op dit moment nog slecht behandelbare, aandoeningen. Het maatschappelijk belang is daarom groot.
De DEC komt tot de conclusie dat de doeleinden van het project het voorgestelde gebruik van de proefdieren en het daarmee samenhangende ongerief van de proefdieren rechtvaardigt.

E. Advies

1. Advies aan de CCD
   - ✓ **De DEC adviseert de vergunning te verlenen**
2. Het uitgebrachte advies is gebaseerd op consensus.
3. Er zijn geen knelpunten of dilemma's gesignaleerd tijdens het beoordelen van de aanvraag of het formuleren van het advies.

15

[redacted]

Van: secretariaat DEC [redacted]
Verzonden: maandag 22 juni 2015 10:08
Aan: ZBO-CCD
Onderwerp: AVD-801002015125 ingediend

Categorieën: [redacted]

Geachte CCD,

Als secretaris van de DEC-KNAW heb ik zojuist alle documenten behorende bij AVD-801002015125 naar u gestuurd via de webftp. Het getekende aanvraagformulier is afgelopen vrijdag per post naar u gestuurd.
Mochten er nog vragen zijn dan hoor ik dat graag.

Groet [redacted]
DEC-KNAW

16

[redacted]

Van: Info-zbo
Verzonden: woensdag 22 juli 2015 17:47
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD801002015125: Aanvullende informatie

Geachte [redacted],

Op 22 juni 2015 heeft de CCD uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project “The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance”. De CCD heeft nog aanvullende informatie van u nodig om uw aanvraag verder te kunnen beoordelen.

-De DEC heeft in haar advies aangegeven dat er enige overlag is tussen de huidige vergunningaanvraag en eerder van een positief advies voorziene DEC protocollen. Om een beeld te krijgen van de mate van overlap en het aantal proefdieren dat daarmee gemoeid is, is het wenselijk antwoord te krijgen op de volgende vragen: In hoeverre is het noodzakelijk dat, daar waar er overlap is, de al eerder van een positief advies voorziene dierproeven en het huidige bij de CCD aangevraagde project naast elkaar uitgevoerd kunnen worden. Indien noodzakelijk, om welke in deze aanvraag beschreven dierproeven gaat het en hoeveel dieren zullen nog gebruikt worden op de DEC protocollen?

-De CCD hecht er aan dat het aantal dieren in voorraad gedood terug te dringen. In uw aanvraag beschrijft u niet of beide geslachten gebruikt kunnen worden. Indien u van plan bent alleen/voornamelijk muizen van 1 geslacht gaat gebruiken, kunt u onderbouwen waarom het belangrijk is dieren van 1 geslacht te gebruiken?

Opsturen informatie
U heeft 14 dagen de tijd om de ontbrekende informatie op te sturen. De CCD zou uw aanvraag echter graag tijdens haar eerstvolgende vergadering behandelen. De CCD zou de gevraagde informatie daarom uiterlijk maandag 27 juli 2015 van u ontvangen. U kunt deze informatie aanleveren via NetFTP of per e-mail.

Wanneer een beslissing
De beslistermijn op uw aanvraag wordt opgeschort tot het moment dat bovengenoemde informatie is ontvangen. Na ontvangst van uw reactie nemen wij uw aanvraag verder in behandeling. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Bij voorbaat hartelijk dank,

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**

Postbus 20401 | 2500 EK | Den Haag

T: 0900 2800028
E: info@zbo-ccd.nl (Let op: nieuw e-mail adres)

Hubrecht Institute

Developmental Biology
and Stem Cell Research

Centrale Commissie Dierproeven
Postbus 20401
2500EK Den Haag

Datum: Utrecht, 24 juli 2015.
Uw referentie: AVD80100-2015-125

Geachte Leden van de CCD,

Deze brief schrijven wij u n.a.v. uw brief/email d.d. 22 juli 2015. In deze brief verzocht u ons om aanvullende informatie nodig om projectvergunning dierproeven (AVD80100-2015-125) verder te kunnen beoordelen. Hierbij voldoen wij aan dit verzoek.

- De DEC heeft in haar advies aangegeven dat er enige overlag is tussen de huidige vergunningaanvraag en eerder van een positief advies voorziene DEC protocollen. Om een beeld te krijgen van de mate van overlap en het aantal proefdieren dat daarmee gemoeid is, is het wenselijk antwoord te krijgen op de volgende vragen: In hoeverre is het noodzakelijk dat, daar waar er overlap is, de al eerder van een positief advies voorziene dierproeven en het huidige bij de CCD aangevraagde project naast elkaar uitgevoerd kunnen worden. Indien noodzakelijk, om welke in deze aanvraag beschreven dierproeven gaat het en hoeveel dieren zullen nog gebruikt worden op de DEC protocollen?

Antwoord vraag 1:
Om de continuïteit van ons onderzoek te garanderen en omdat een aantal op dit moment lopende goedgekeurde DEC protocollen allemaal in een verschillend stadium van uitvoering zijn is het onvermijdelijk dat er een overlap is tussen deze projectaanvraag en de lopende DEC protocollen. Na het verlenen van de projectvergunning door de CCD zullen al onze proefdieren en dierexperimenten formeel gaan vallen onder deze vergunning en zullen de dieren en de experimenten 'afgeschreven' worden van de beschrijvingen en aantallen in projectbeschrijving.

-De CCD hecht er aan dat het aantal dieren in voorraad gedood terug te dringen. In uw aanvraag beschrijft u niet of beide geslachten gebruikt kunnen worden. Indien u van plan bent alleen/voornamelijk muizen van 1 geslacht gaat gebruiken, kunt u onderbouwen waarom het belangrijk is dieren van 1 geslacht te gebruiken?

Antwoord vraag 2:
In onze beschreven experimenten maken we, waar mogelijk, geen onderscheid tussen beide geslachten en zullen zowel mannetjes als vrouwtjes gebruikt worden. Dit zal resulteren in een vermindering van het

Hubrecht Institute

Developmental Biology
and Stem Cell Research

aantal in voorraad gedode dieren. Uitzondering hierop zijn studies van tumoren die geslachts-afhankelijk zijn zoals borstkanker.

Wij hopen U hiermee voldoende geïnformeerd te hebben en zien uw reactie met belangstelling tegemoet.

Hoogachtend,

[redacted]

[redacted] - KNAW

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
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www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015125

Uw referentie

Bijlagen
1

Datum 10-08-2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 22 juni 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance" met aanvraagnummer AVD801002015125. Wij hebben uw aanvraag beoordeeld.

Op 24 juli 2015 heeft u uw aanvraag aangevuld na vragen van het secretariaat.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de dierproeven (hierna de wet), voor de periode van 12 augustus 2015 tot 01 juni 2020. Hierbij gelden de voorwaarden zoals genoemd in de vergunning. De looptijd van de vergunning wijkt af van de aangevraagde periode omdat de aangevraagde startdatum van het project in het verleden ligt. U kunt met uw project "The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance" starten.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC KNAW gevoegd d.d. 22 juni 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. Naar aanleiding van de door de DEC gesignaleerde overlap tussen de huidige vergunningsaanvraag en eerder van een positief advies voorziene DEC protocollen, is de voorwaarde toegevoegd dat daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen de dieren en experimenten na het verlenen van de vergunning formeel onder deze vergunning gaan vallen, zoals u in uw brief van 24 juli 2015 heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

Het DEC advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze gegevens in het colofon.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: KNAW
Adres: Postbus 19121
Postcode en woonplaats: 1000GC Amsterdam
Deelnemersnummer: 80100

deze projectvergunning voor het tijdvak 12 augustus 2015 tot 01 juni 2020, voor het project "The molecular and cellular mechanisms of tumor initiation, growth, metasasis and therapy resistance" met aanvraagnummer AVD801002015125, gebaseerd op het advies van Dierexperimentencommissie DEC KNAW.
De functie van de verantwoordelijk onderzoeker is Group Leader.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 22 juni 2015
2. de bij het aanvraagformulier behorende bijlagen:
    a. Projectvoorstel, zoals ontvangen bij digitale indiening op 22 juni 2015 en brief ontvangen op 24 juli 2015;
    b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 22 juni 2015;
    c. Advies van Dierexperimentencommissie d.d. 22 juni 2015, ontvangen op 22 juni 2015;
    d. Aanvullende informatie ontvangen op 24 juli 2015.

Dierproeven

Naam dierproef	**Diersoort**	**Aantal dieren**	**Ernst**
Gene (in) activation interventions (ex vivo analysis)	Muis (WT, GGM en mutanten) Embryo's Neonaten Volwassenen	 200 100 4000	 Licht Licht Licht: 96% Matig <1 dag: 1% Matig continu: <3%
Gene (in) activation interventions (in vivo imaging)	Muis (WT, GGM en mutanten) Volwassenen	 5000	 Licht: 15% Matig: 85%
Transplantation (ex vivo analysis)	Muis (WT, GGM en mutanten) Volwassenen	 4500	 Licht: 96% Matig <1 dag: 1% Matig continu: <3%
Transplantation (in vivo imaging)	Muis (WT, GGM en mutanten) Volwassenen	 2000	 Licht: 12% Matig: 88%
Transplantation Gene (in) activation interventions (ex vivo analysis)	Muis (WT, GGM en mutanten) Volwassenen	 1650	 Licht: 96% Matig <1 dag: 1% Matig continu: <3%
Transplantation Gene (in) activation interventions (in vivo imaging)	Muis (WT, GGM en mutanten)		

	Volwassenen	2800	Matig
Generation Welfare assessment and Breeding of GMM	Muis (WT en GGM) Volwassenen: Donoren Foster dieren GGO dieren Immuun deficiënte dieren Verklikkerdieren	2260	 Matig Matig Geen: 99% Licht: 1% Geen Geen

Voorwaarden
Op grond van artikel 10a1 lid 2 Wet zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen zullen de dieren en experimenten na het verlenen van de vergunning formeel onder deze vergunning gaan vallen, zoals de aanvrager in zijn brief van 24 juli 2015 ook heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

In artikel 10, lid 1a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Weergave wet- en regelgeving

Dit project en wijzigingen

Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging

De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn.
In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving

In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef

Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.

Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.

	Inventaris Wob-verzoek W16-01								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 2015126								
1	Aanvraagformulier				x		x	x	
2	Niet-technische samenvatting	x							
3	Projectvoorstel				x			x	
4	Bijlage beschrijving dierproeven 1			x					
5	Bijlage beschrijving dierproeven 2			x					
6	Bijlage beschrijving dierproeven 3			x					
7	Bijlage beschrijving dierproeven 4			x					
8	Bijlage beschrijving dierproeven 5			x					
9	Flow chart				x		x	x	
10	Overzicht aantallen				x		x	x	
11	DEC-advies				x		x	x	
12	Ontvangstbevestiging				x		x	x	
13	Mail indienen 15-7-2015				x		x	x	
14	Mail aanvraag 20-7-2015				x		x	x	
15	Mail vervolgbrief 31-7-2015				x		x	x	
16	Vervolgbrief				x		x	x	
17	Acceptatiebrief				x		x	x	
18	Mail aanvullende informatie 10-8-2015				x		x	x	
19	Brief aanvullende informatie				x		x	x	
20	Beschikking				x		x	x	
21	Vergunning			x					
22	Mail beschikking 13-8-2015				x		x	x	
23	Advies CCD		x						x

16 JULI 2015

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.zbo-ccd.nl of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☑ Ja > *Vul uw deelnemernummer in* 80101 Nederlands Herseninstituut-KNAW 1126

☐ Nee > *U kunt geen aanvraag doen*

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	KNAW
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	5 4 6 6 7 0 8 9

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	
Postbus	Postbus 19121
Postcode en plaats	1000GC Amsterdam
IBAN	NL33DEUT0546900054
Tenaamstelling van het rekeningnummer	Nederlands Herseninstituut

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	Group Leader
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	☑ Dhr. ☐ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☑ Dhr. ☐ Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☑ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☑ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☑ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	0 1 - 0 8 - 2 0 1 5
		Einddatum	0 1 - 0 8 - 2 0 2 0
3.2	Wat is de titel van het project?	Neurobiology of compulsive behavior and its components: Brain stimulation and ...	
3.3	Wat is de titel van de niet-technische samenvatting?	Compulsief gedrag en zijn componenten: neurobiologische metingen en hersenstimulatie	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC-KNAW
		Postadres	[redacted] Amsterdam
		E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☑ Nieuwe aanvraag Projectvergunning € 741,00 Lege

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

☐ Via een eenmalige incasso

☑ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☒ Appendixen 5 maal; flow chart

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.6). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted]
Plaats	Amsterdam
Datum	10-07-2015
Handtekening	[redacted]

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101 – Nederlands Herseninstituut-KNAW
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW
1.3	Provide the title of the project.	Neurobiology of compulsive behavior and its components: Brain stimulation and measurements

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

The term compulsivity is used to describe dysfunctional behavior in various neuro-psychiatric disorders such as substance and behavioral addictions, obsessive-compulsive disorder (OCD), impulse-control disorders, and eating disorders. The presence of compulsive tendencies in all of these disorders makes a

strong case for a shared underlying mechanism. Compulsive behavior is characterized by the feeling that one 'has to' perform a specific act as a result of an urge (e.g., to avoid a negative emotional state). At the same time, patients are aware of the conflict between the performed act and maintaining their quality of life. Thus, compulsivity produces behavior that we perform against our will and despite its negative consequences. Examples of such consequences are the OCD patient unable to hold a job because he/she spends almost every waking hour cleaning his/her house or the addict that isolates him/her-self from friends and family because he/she continues to relapse to drug abuse despite the best intentions not to. It has been suggested that compulsivity is a so-called 'endophenotype', a behavioral pattern or response mode that is preserved across different disorders. In order to better characterize this concept, its composition can be described as separate components: 1) persistence of performance, 2) elicitation of undesirable consequences, 3) escalation of symptoms over time, 4) aggravation by stress and anxiety, 5) exaggerated habit formation, 6) response inflexibility, and 7) loss of voluntary control. Whereas 1), 2), 3) and 7) are components that cannot be studied separately from the compulsive phenotype itself, 4), 5) and 6) may be endophenotypes that exist in the normal population and lower the threshold for compulsive behavior to develop. **For a better understanding of disorders featuring compulsivity, both the obvious pathological phenotype (compulsivity) itself, as well as these individual components have to be investigated**.

One prominent theory of compulsive behavior proposes a dysfunction or imbalance between competing brain systems: the system that controls more purposeful, goal-directed behavior and the system that supports automatic, habitual behavior (Everitt and Robbins, 2005; Dalley et al, 2011). Goal-directed behavior is based on knowledge of the causal relationship between an action and its consequences (outcomes), and is only performed when those consequences are desired. In contrast, habitual behavior consists of actions that are automatically triggered by environmental stimuli regardless of the current desirability of the consequences, insensitive to its outcomes. These stimulus-response associations that mediate habitual behavior have been strengthened either by reward (positive reinforcement; e.g., drug abuse that improves the user's mood) or by the omission of an aversive event (negative reinforcement; e.g., house cleaning in order to avoid feeling anxious). Habitual behavior can arise under many conditions, the most common one of which is extensive behavioral repetition (Dickinson et al, 1985). However, habits can also arise from failures in goal-directed control and exposure to stress, which can lead to habitual behavior even without extensive behavioral repetition, underlining the competing hierarchy between these behavioral control systems. Therefore, **both systems, habit and goal-directed, can contribute to the likelihood that a habit will be formed in a given situation**.

In patients suffering from psychiatric disorders with compulsivity, such as OCD and drug addiction, an increased tendency towards forming habits has been reported (i.e., in these individuals habits form faster and the habit system dominates their behavior; Voon et al, 2014; Gillan et al, 2015), which has been observed regardless of whether such habits work toward gaining reward or toward avoiding punishment. Stress and anxiety, states present in many disorders, may contribute to this enhanced habit formation (Schwabe et al, 2011). Moreover, it has been suggested that in such a situation habits can become excessive and progressively compulsive as a result of disturbances of brain systems controlling behavior (e.g., a drug-use habit escalates into compulsive drug addiction or cleanliness escalates into obsessive-compulsive cleaning (escalation)). However, how far aberrant habit formation contributes to compulsivity is currently still unclear. Also unclear is whether hypo-function of the goal-directed or hyper-function of the habitual system drives the exaggerated tendency to display habits, and the extent of stress and anxiety as driving forces. Importantly, **despite the habit-hypothesis being the most prominent contemporary theory, dysfunction of any or all of the other individual components mentioned above** (e.g., response inflexibility or insensitivity to negative consequences etc.) **are alternative sources of compulsive behavior** that may or may not interact with habits to produce pathology. Thus, for a better understanding of disorders featuring compulsivity and in order to answer the following questions, both the compulsive behavior itself ('models') as well as all individual components potentially contributing to compulsivity and their interaction have to be investigated. How are these components related to each other? Are habit-prone individuals more susceptible to develop compulsivity? Do habits escalate into compulsivity? Is compulsivity an "endophenotype" that is linked to genetic variations in neurobiological processes? Or are these patients just unable to flexibly change or lose voluntary control of their behavior? And importantly: What are the underlying neurobiological mechanisms of these processes?

The basal ganglia, a subcortical brain system found in all mammals, are thought to serve the purpose of selecting a behavior strategy appropriate for a given situation through interaction with cortical regions that participate in higher cognitive processes and sensorimotor performance. The main input nucleus of

the basal ganglia, the striatum, receives inputs from different functional units of the cortex, and the neurotransmitter dopamine acts as a regulator of cortical information flow through the striatum. Several regions of the cortex and the striatum are thought to be involved in controlling goal-directed and habitual behavior. For example, the medial striatum and the medial prefrontal cortex (mPFC) have been shown to subserve learning involving goal-directed behavior. In contrast, the dorsolateral striatum (DLS) and the infralimbic cortex (IFC) are necessary for the formation of habits. Dysfunction of these systems is indicated in several compulsive disorders: 1) Drug addiction and OCD are characterized by altered activity of the dopamine system, which could manifest as a tendency for natural rewards to lose their value. 2) Dysfunctions of the striatum and mPFC are present in addiction, OCD, obesity, and bulimia nervosa (Robbins et al, 2011). In summary, **different 'cortico-striatal loops' are putatively associated with aspects of compulsivity** but it is still unclear which specific brain systems mediate this development and how they interact.

Although a variety of behavioral and pharmacological treatments for compulsive disorders are available, in many cases such therapy is not successful. Thus, it is of great importance to identify new targets and to develop more effective therapies. A promising avenue opened up when it became clear that **deep-brain stimulation (DBS) is effective in a number of psychiatric disorders**. Although it is not exactly clear how this high-frequency electrical stimulation exerts its effects, recent data suggests that it may interrupt the pathological activity of a cortico-striatal loop, allowing an attentional shift away from excessive processing of disease-related, habit/compulsivity-eliciting stimuli and restoration of goal-directed behavior. Thus, stimulation of a relatively small target area may potentially lead to rapid, broad, and clinically relevant changes in brain network function. However, as said, our understanding of the mechanisms of action of how DBS reduces compulsivity is still limited. What is the neurobiological basis of the therapeutic effects of high-frequency stimulation in this spectrum of compulsive psychiatric disorders? What are the best and safest brain targets for changing pathological behavior in OCD, addiction, and eating disorders? Thus, another long-term goal of our research line is to contribute to answering such questions. As these disorders are characterized by profound behavioral alterations due to disturbances of affect, motivation and cognition, our research proposed in this application is focused on neurobiological substrates of affect, motivation and cognition in general as well as specific key features (components; see above) of these disorders, such as for example the nature of inflexible behavior.

The main objective of the project is to provide a better understanding of the neurobiology of compulsive behavior. In order to do so, compulsive behavior itself AND a variety of motivated behaviors thought to constitute or contribute to compulsivity (components) will be studied in rodents while neural measurements and interventions are performed.

How to study compulsive behavior in rodents: It is difficult to model all aspects of a psychiatric (human) disorder in animals, but it is feasible to study certain aspects of such psychiatric disorders, focusing on distinct neurobehavioral domains. Human compulsive behavior has many aspects and it is still not clear whether it is a "single endophenotype" with a single underlying mechanism, or whether different forms of compulsivity with different mechanisms exist. Thus, to properly model compulsivity in animals, the use of several models is necessary. Animal models for compulsive disorders can for example model the progression from recreational to compulsive consumption of drugs of abuse or high-fat/high-sugar foods. Furthermore, the development of excessive repetitive performance of purposeless actions, akin to OCD, can for example be studied in the Sapap3-mutant mouse model, which shows excessive grooming behavior associated with increased levels of anxiety (Welch et al, 2007). In a more recent model, mice develop increased grooming behavior after optogenetic stimulation of a corticostriatal brain circuit (Ahmari et al, 2013). Another, pharmacological paradigm allows the study of the development of compulsive licking and/or checking behavior in rats, following repeated administration of quinpirole, a dopamine receptor agonist (Eagle et al, 2014). Finally, compulsive drinking may develop in animals exposed to repetitive timed food presentation (schedule-induced polydipsia (Falk, 1961). Common read-outs of these compulsive behaviors are the registration of natural repetitive actions (grooming, licking). Additional tests may be used to probe the presence of perseverative, inflexible and/or habit-like behavior in operant tasks where an animal may obtain a reward or may avoid a punishment. A special form of this is the signal attenuation model, in which rats are tested in a condition of diminished response feedback (Joel, 2006). In this way, the relation between excessive repetition of natural behaviors likely to establish habits and the presence of compulsivity in more complex, acquired behaviors is studied (including for example cognitive flexibility). At the same time, these models are perfectly suited to study the underlying neuronal mechanisms and test novel treatment strategies. In order to answer the question whether compulsivity is a single 'endophenotype', a behavioral response mode that is preserved across environmental contexts and disorders, it is necessary to use a number of different animal models of

compulsivity. Verification of whether this trait is stable across different behavioral tests and whether the same underlying brain mechanisms can be identified in these tests, will enable the exploration of this question. We hypothesize that there is indeed a single compulsivity trait.

Our group has gathered extensive experience in using various models of compulsive behavior, most notably with compulsive self-administration of cocaine in rats, compulsive grooming in Sapap3-mutant mice, compulsive checking in quinpirole-treated rats, and compulsive responding following "signal attenuation" in mice. In our lab, operant boxes, various mazes, and other test arenas are successfully employed to analyze spontaneous and learned behavior, including habit formation, reversal learning and other tests of cognitive flexibility. These rodent models are routinely combined with *intervention techniques*, such as optogenetic intervention, DBS, intracerebral and systemic drug administration, and *measurements of brain activity,* such as neurochemical and neurophysiological methods including microdialysis, fast-scan cyclic voltammetry, and electrophysiology. Our results showed for example, that dopamine release in the brain is different between rats progressing to compulsive cocaine self-administration and rats that do not. We also found that differences in dopamine signaling in the striatum are predictive for the ability to rapidly learn a reversal of response-reward relations. Furthermore, neuronal activity measured in the striatum and connected cortical regions was predictive of compulsive behavior after repeated quinpirole administration. Based on this experience, we feel that we can take the next step in our research and try to answer the question which common neurobiological mechanisms underlie the development, escalation, and persistence of different forms of compulsive behavior and which brain stimulation targets may contribute to remission of compulsive behavior. To our knowledge, this approach is unique – worldwide, most groups work on a single model of compulsivity, most often substance addiction. Moreover, attempts to reverse compulsive behavior using DBS are still scarce and, if performed, are not followed up by studies of the underlying mechanisms. We are among the few groups worldwide where direct and extensive interactions between animal and clinical scientists is practiced.

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Welch et al (2007) Cortico-striatal synaptic defects and OCD-like behaviors in Sapap3-mutant mice. Nature Aug 23;448 (7156):894-900.

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

As explained above, compulsive behavior is believed to be a central common denominator to several neuro-psychiatric disorders such as addictions, obsessive-compulsive disorder (OCD), and eating disorders. Compulsive behavior is probably constituted by a number of individual components, such as behavioral inflexibility, and it is aggravated by stress and anxiety, whereby it is hypothesized that

aberrant habit formation is crucial for its development. The main objective of the project is to provide a better understanding of the neurobiology of compulsive behavior. In order to do so, compulsive behavior itself and a variety of its presumed components will be studied while neural measurements and interventions are performed.

The general research questions addressed by this approach are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

These objectives are achievable because...
1) … the lab has powerful research tools at its disposal (e.g., opto- and chemogenetics, electrophysiological and electrochemical detection of brain activity, animal models for compulsive disorders),
2) … the lab is situated in an excellent research environment with state-of-the-art facilities and close proximity to outstanding scientists at [redacted],
3) … the lab has outstanding national and international collaborators (e.g., [redacted]),
3) … the proposed strategy is heavily grounded on our laboratory's expertise,
4) … technical skills necessary are almost all already in place,
5) … we have a close collaboration with [redacted] warranting proximity to the clinical condition
6) … we have a proven track record of successfully conducting and publishing comparable research in highly respectable scientific journals in the past (e.g., [redacted]).
7) … we have a proven track record of successful grant applications (e.g., [redacted])

References:

- [redacted]
- [redacted]
- [redacted]
- [redacted]
- [redacted]

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

Scientific relevance
The identification, characterization, and understanding of brain pathways mediating a progression from learning a new behavior to its automatic execution and in some cases to pathological compulsive execution, will have great impact on several fields of neuroscience. Such pathways, although assumed for decades, have yet to be verified. All fields that focus on subjects related to the translation of motivation into behavioral action will be affected, including research either restricted to motor systems only or fields exclusively focused on motivation and emotion. Furthermore, this application is aimed at further elucidating a long-standing question in the field - if and how habits, compulsions, behavioral flexibility, stress, anxiety and impulsivity are related to each other. Thus, discovery of such brain pathways and their functional relevance will potentially offer fundamental insights into how we/our brains control behavior.

Social relevance
Compulsivity research is expected to lead to novel insights that may tie psychiatric diseases together that are now often studied and treated separately, and paving the way to define and treat conditions that

are common to obsessive-compulsive spectrum disorders, substance addiction and eating disorders. *Knowledge and understanding of the neurobiological mechanisms of conditions such as exaggerated habitual responding, inflexible behavior or high sensitivity to stress will lead to a better understanding and hopefully an improved acceptance of psychiatric patients in our society.* This knowledge will also be applied in clinical psychiatric research to improve and extend treatment options for individual patients and for the society as a whole. DBS is one of these treatment options, as its application has been successfully extended from neurological disorders to mental health conditions, a relatively new field that still needs improvement. Thus, our results could be of great benefit for psychiatric conditions by identifying new target brain regions for DBS electrode placement to improve therapeutic efficiency (e.g., specific anatomical circuitry underlying maladaptive habits). The lab's close ties with the [redacted] favors the realization of such future DBS applications. For example, if we are able to identify a specific brain pathway that is crucial for rendering inflexible behavior flexible, DBS electrodes could be directed at that region in OCD patients, addicts, and other patients in a clinical trial. Furthermore, stimulation parameters could be optimized in animal models of compulsive disorders. Together, these approaches may improve the quality of life of many different patient populations. Finally, technological advances such as DBS electrodes that can record brain activity in addition to provide stimulation may lead to closed loop, feedback-based appliances, that cannot be developed and applied without thorough testing in experimental animals.

Translational relevance
Our findings will be communicated to basic scientists, clinicians, clinical researchers, and the general public. We will take advantage of the [redacted], operated by professional journalists. We have previously utilized these services for one of my publications in [redacted] in 2014 in order to engage the general public through Dutch media nationwide (e.g., [redacted]). Such outreach is of importance to raise awareness for the problems of patient suffering from compulsivity, but also to support patients by explaining the biological foundation of their troubles to them.
Although the research proposed in this application is fundamental in nature, the knowledge gained from these studies has great potential for translational utilization. An important contributing factor to enable this is the embedding of my [redacted] research group in a larger clinical research team at the [redacted], where we are involved in weekly meetings. These close ties provide optimal conditions to set up translational, multidisciplinary research.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

We are interested in how the brain controls behavior. The ultimate, long-term goal is to better understand how processes mediating learning, emotion, and motivation are affected in pathological conditions such as compulsive behavioral disorders and to invigorate the development of new treatment strategies and optimize existing therapies. We have strong ties with clinical researchers studying compulsive behavior, its neurobiological characteristics and its therapies in patients (in particular DBS). To contribute to the understanding of such pathological processes, we use rodent models of compulsive behavior. By choosing a variety of models we aim to discover the shared mechanism in the various presentations of compulsive behavior. To fulfil this aim, we chose several models that reflect sufficiently different phenotypical presentations: Pharmacological (e.g. repeated administration of the dopamine agonist quinpirole leads to compulsive checking and loss of flexible responding), genetic (e.g. Sapap3-mutant mice show increased anxiety and develop compulsive grooming) and addiction models (e.g. extended exposure to self-administration of cocaine leads to compulsive seeking and taking of the drug). Together, they model compulsive behavior from simple repetitive actions (grooming; reminiscent of autism or impulse disorders) to complex behavior (checking and drug seeking; reminiscent of obsessions and substance addiction). The use of mice in addition to rats is based on the fact that transgenic mice such as the Sapap3-mutant mice are up to now the only model with spontaneous compulsive behavior, where no additional pharmacological treatment or behavioral training is required. These are also the models that we have been using for 2-6 years now and, therefore, form our first tier of models, but these models may be extended or replaced by other pharmacological, behavioral and addiction models, that e.g. show a less variable presentation.

These models will be used to study the development of compulsivity, the individual variation of the phenotypes and the behavioral nature of the dysregulation in the first part of the project (**3.4.4.1 Establishing and characterizing rodent behavior that models compulsive behavior and its**

components; see flow chart – the main read-out is behavior). Compulsivity in patients is regarded as behavior performed despite negative consequences. The animal models should incorporate similar negative consequences (e.g. the skin lesions developing in Sapap3-mutant mice as a result of excessive grooming or the foot shocks cocaine-addicted animals are prepared to accept to obtain their drug reward). Furthermore, we are interested in the presence of the various components of compulsivity, such as increased habit formation, diminished cognitive flexibility, and anxiety and aggravation by stress. These components are important as they may represent "building blocks" of a general compulsive phenotype. One of the outstanding questions is whether they are present before compulsivity develops, or whether they may be consequences of that. Our hypothesis is that they are present as predisposing factors that lower the threshold for the development of compulsive activities. In parallel experiments, our clinical collaborators also study the relation between components such as habit formation and overall OCD symptoms. Thus, similar approaches allow translational comparisons. Together, in 3.4.4.1 we will improve behavioral tests but also use these tests to answer questions such as how does compulsive behavior develop and what is the relation with separate behavioral components?

In the subsequent parts of the project, we will focus on the study of the neurobiological mechanisms underlying the behavior (**3.4.4.2 Identification of brain correlates of compulsive behavior and its components**; see flow chart). Here, both (1) normal function of brain circuits responsible for the production of functional behavior, as well as (2) the nature of dysregulation in the "diseased" state have to be investigated. We aim to do so by employing a variety of techniques to measure brain activity and neurotransmitter release in awake rodents. Our prime interest here is to characterize the functional status of the cortico-basal ganglia-thalamic circuits that support normal motivated behavior and are thought to be dysregulated in the case of compulsive behavior. These measurements will allow us to detect neurobiological correlates of compulsive actions (e.g., grooming bouts in transgenic mice; compulsive lever-pressing for drug-related cues in rats).
Results will be discussed with our clinical colleagues and compared with the clinical results that are obtained using various measuring techniques.

In parallel, to discover causal relationships between brain activity and behavior, brain activity is manipulated (**3.4.4.3 Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation**; see flow chart) using different interventions such as DBS, pharmacogenetics, optogenetics, lesions, and pharmacological treatments (the main read-out is behavior). The target position will be based on the knowledge we have of the functional circuits supporting the compulsivity and its components and in normal animals.

Finally, the interplay of neurobiological variables (e.g., neurotransmitters) and neuroanatomical pathways are investigated during behavior (**3.4.4.4 Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation** – main read-out is the relation between behavior and neuronal activity) but also in the anesthetized animal or brain slices, where, in isolation, brain activity is easier to assess and brain stimulation is applied easier (**3.4.4.5 Identification and characterization of neuroanatomical connections and their regulation**; see flow chart – the main read-out is neuronal activity).
Of these two possibilities, the combination with behavior may be considered the highest level of integration. These studies are restricted to the situation where an intervention has resulted in a promising behavioral effect (e.g. anticompulsive effect in one or more of the models) and information on the neurobiological mechanisms of action is wanted.

The experiments under anesthesia (terminal studies) may be applied to obtain information on circuit connections, selectivity of targeted interventions, etc. Thus, the functional status of the corticostriatal pathway in Sapap3-mutant mice may be studied by electrical or more selective optogenetic stimulation of prefrontal areas and electrophysiological recording or Ca-imaging of striatal activity; The choice for such experiments under anesthesia is also driven by the wish to limit discomfort and only use awake animals in experiments that involve behavior or measurements that are known to be fundamentally affected under anesthesia.

Choices and Go/NoGo decisions.
The choice for the first three animal models for compulsive behavior (first tier) is based on their different characteristics that will allow us to discover the shared mechanisms in the various presentations of compulsive behavior. Further model choices in the course of the project will depend on scientific, practical, and animal-welfare factors. The choice is always determined by the wish to have the best practical option to answer our main scientific questions, with the least discomfort for the animals. Once a

behavioral strategy is established and the desired behavior is reproducibly detected (3.4.4.1), brain activity during this behavior is assessed in another set of animals (measurement; 3.4.4.2) or interfered with in another set of animals (intervention; 3.4.4.3) in order to unravel the neurobiological underpinnings of this behavior (GO point). If we are not able to obtain reproducible and validated compulsive behavior (3.4.4.1) such neurobiological measurements (3.4.4.2) or interventions (3.4.4.3) will not be attempted (NOGO point). If we yield promising results, a combination of the two will be attempted (optional GO point; 3.4.4.4), otherwise not (NOGO point). Thus, in summary, we will only start using an animal model in 3.4.4.2 and 3.4.4.3 after this model has been implemented successfully under 3.4.4.1, and we will only start 3.4.4.4 following successful execution of 3.4.4.2 or 3.4.4.3.
An additional approach is the combination of neurobiological measurements and interventions in the anesthetized animal or in brain slices (3.4.4.5). Here, circuits or targets that are of interest are directly studied without preceding behavioral studies. In case of a positive outcome, these targets may be used in the combined studies of 3.4.4.2 and 3.4.4.3, and may eventually be followed by 3.4.4.4.

Before we start our experiments we will write an application to the IvD. In this application we will exactly describe (among others) which considerations, facts and results have led to the proposition of the experiments, which specific question(s) we are trying to answer with the proposed animal experiments and what the ultimate goal is for the proposed experiments. Moreover we will describe in full detail the experimental design, discuss the number of animals in the experiments, describe human endpoints, alternatives, and the nature of discomfort. Experiments will only be started upon IvD approval.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

3.4.4.1 Establishing and characterizing rodent behavior that models compulsive behavior and its components

Behavioral assays are carried out in standard operant boxes (e.g., levers that can be pressed by the animal to achieve an outcome), touch-screen boxes (touch screens instead of levers), open-field boxes (empty arenas for animals to explore), and mazes of different shapes (more complex arenas animals can explore and find rewards). In these rodent assays (1) models of compulsive behavior; and (2) components of compulsive behavior, among which habit formation, cognitive flexibility, the relation with fear and anxiety and the impact of stress or enrichment are assessed. Assays that will be employed will probe spontaneous behavior, reward-driven behavior, and also punishment-driven behavior. Depending on the model and the additional component(s) studied, the total testing time for individual animals will vary between 2 and 6 months, but transgenic animals spontaneously showing compulsive behavior may be followed for up to 1 year. Important additional (e.g. correlational) information is expected from testing animals in more than one behavioral assay, one of which is assessing the compulsive behavior. Thus, e.g. anxiety and reward-driven behavior may be characterized before an animal is made compulsive by repeated quinpirole administration; cognitive flexibility is tested in an animal before and/or after being trained to compulsively self-administer cocaine; a Sapap3-mutant mouse that shows compulsive grooming is studied in a habit-formation paradigm, etc.

The general organization is:
A) to establish and validate animal *models* for compulsive behavior – in pilot experiments a satisfactory paradigm is selected for further studies.
B) to characterize the tested population of animals (mice and rats) for individual differences to unravel biological mechanisms.
C) to establish and validate behavioral methods to assess *components* of compulsive behavior – in pilots a satisfactory paradigm is selected.
D) to characterize the compulsivity in relation to its behavioral components – based on the paradigms developed in C, experiments in which one of the components is tested in one of the models of compulsive behavior. All these experiments will also include the testing of general anxiety as a second component tested.

We will start in A) with 3 models (Sapap3-mutant, cocaine self-administration and quinpirole treatment) and characterize individual differences in B). We will start in C) with habit formation and combine that in D) with the three models.

Aspects of this section that will extend beyond pure behavioral testing are:
a. Drug self-administration will require catheter implantation in order to apply intravenous drug injection.
b. Tests to establish "true" compulsivity in rewarded behavior will involve testing behavior in combination

with foot shocks and acquired taste aversion.
c. Sapap3 mutant mice can develop a phenotype with constitutional discomfort. Close monitoring prevents development of skin lesions with more than moderate discomfort.
d. Impact of acute or chronic stress on behavior and well-being will be monitored by measurement of plasma levels of e.g. corticosterone.

3.4.4.2 Identification of brain correlates of compulsive behavior and its components

Once a behavioral strategy is established and the desired behavior is detected, brain activity during this behavior is measured in order to unravel the neurobiological underpinnings of this behavior. Thus, in these procedures behavioral tests described under 3.4.4.1 will be combined with a "neuro-measurement" technique. The techniques to measure brain activity in awake rodents in our lab are:
Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity (virus injections prior to testing necessary to visualize calcium release)
Measure-5) functional magnetic resonance imaging (fMRI) to assess whole-brain activity

The general organization is:
A) to establish and validate the measurement techniques in animal *models* for compulsive behavior – a paradigm is selected (in 3.4.4.1).
B) to measure the neuronal activity parameter in the animal *models* –will deliver data of neuronal activity during compulsive behavior.
C) to establish and validate the measurement techniques when *components* of compulsive behavior are studied in animals showing compulsive behavior – in pilot experiments a satisfactory paradigm is selected.
D) to measure the neuronal activity parameter in the animal models while they are engaged in one of the *components* –will deliver data of neuronal activity during e.g. habit formation in compulsive animals.

Depending on the results with 3.4.4.1 we will start in A) with three models (e.g. a transgenic mouse, a rat pharmacological and a rat addiction model) and three measurement techniques (electrophysiology, fast-scan cyclic voltammetry and fMRI). Electrophysiology and voltammetry will be used in all three models, but fMRI will be restricted to the rat models. We will then perform formal measurements under B). We will start in C) adding the component that in 3.4.4.1 turned out to be the most interesting one – formal measurements are then carried out under D).

All of the above measuring techniques (3.4.4.2) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement. Measure-1 through Measure-4 require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays. Measure-5 requires head re-straining because movement artefacts will otherwise prevent measurements. The use of behavioral tests in combination with Measure-5 is restricted and will mainly concern inducing a compulsive phenotype before measurements with fMRI.

3.4.4.3 Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation

To discover causal relationships between brain activity and behavior, behavioral tests described under 3.4.4.1 will be combined with a "neuro-intervention" technique, which will often involves invasive procedures. The techniques to interfere with brain activity in awake rodents in our lab are:
Intervent-1) deep-brain stimulation (DBS; high-frequency electric stimulation of brain tissue via intra-cranially implanted micro-electrodes)
Intervent-2) pharmacogenetics (virus injections prior to testing necessary for expression of drug-sensitive receptors in specific cell populations and subsequent intervention by drug application)
Intervent-3) optogenetics (virus injections prior to testing necessary for expression of light-sensitive receptors in specific cell populations and subsequent intervention by light application)
Intervent-4) brain lesions (both permanent and transient inactivation of specific brain regions via intracranial injection of substances)
Intervent-5) pharmacological treatments (both systemic and intracranial application of neuro-active drugs)

The general organization is:

A) to establish and validate the intervention techniques in animal *models* for compulsive behavior –a satisfactory paradigm is selected (in 3.4.4.1).
B) to interfere with the neuronal activity in the animal *models* –will deliver behavioral data of intervention-induced alterations in compulsive behavior.
C) to establish and validate the intervention techniques when *components* of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory paradigm is obtained; that paradigm will be validated.
D) to interfere with the neuronal activity in the animal models while they are engaged in one of the *components* –will deliver behavioral data of intervention-induced alterations in habit formation etc in compulsive animals.

Depending on the results with 3.4.4.1 and 2 we will start in A) with 3 models (e.g. a transgenic mouse, a rat pharmacological and a rat addiction model) and 3 intervention techniques (DBS, optogenetics, pharmacogenetics). We will then perform formal experiments under B).
We will start in C) with the component that gave the most promising results in the previous studies and combine that with the three models.

Most of the above techniques (3.4.4.3) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement and tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays.

3.4.4.4 Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation
Based on the results and/or the experience with behavioral and neurophysiological/neurochemical measurements, and with neural interventions, we will use combinations of one of the neuro-intervention with one of the neuro-measurement techniques described under 3.4.4.2 and 3.4.4.3 in ongoing behavior, as described under 3.4.4.1. Such combinations will allow the assessment of whether manipulated brain activity takes place, and whether it manifests time-locked to the ongoing behavior. When the results obtained in the measurement and/or intervention section that combined experiments are indicated, the general organization is:
A. to establish and validate the combination of intervention and measurement techniques in animal models for compulsive behavior, either with or without engagement in one of the components – in pilot experiments a satisfactory paradigm is selected for further studies.
B. to interfere with the neuronal activity in the animal models and measure the effect on neuronal activity – will deliver causal relations between intervention-induced alterations in behavior and neuronal activity.

3.4.4.5 Identification and characterization of neuroanatomical connections and their regulation
In a parallel approach, the interplay of neurobiological variables (e.g., neurotransmitters) and neuroanatomical pathways are investigated in the anesthetized animal, where, in the absence of movement, brain activity is easier to assess and brain stimulation is applied easier (no long-term implantation necessary). Questions that can be answered with this approach include: How do the basal ganglia propagate information (neuronal activity) between different functional subunits? Or how reactive are different subregions to neuronal input from the cortex or the midbrain (inputs activated with a stimulation technique (e.g., optogenetics and output measured with a measuring technique (e.g., voltammetry). Thus, in anesthetized animals (short-term experiments → no survival surgery), we will combine the use of one of each of the under 3.4.4.2 and 3.4.4.3 described neuro-intervention and neuro-measurement techniques. However, in the case of optogenetic interventions, this would have to be preceded by stereotactic micro-infusion of a virus to locally express light-sensitive or other proteins.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

The coherence between the above outlined components stems from the overarching goal to identify and characterize neural substrates for compulsive behavior and its "building blocks" (i.e., provide a better understanding of the neurobiology of compulsive behavior; **see flow chart**). Specifically, in each component either behaviors or measures of brain activity that are associated with compulsivity are studied. In order to do so, a variety of motivated behaviors thought to constitute or contribute (or both) to compulsivity will be studied while neural recordings and stimulations are performed. All components

taken together are capable of accomplishing this goal. Thus, the primary logical structure of the approach is 1) to establish a specific rodent behavior of interest, then 2) to identify and measure brain regions that may be involved, then 3) to manipulate that brain region and assess its effect on behavior, and finally either 4) to assess its effect on other brain variables *during* behavior, or 5) in anesthetized animals. Although all these components are intended to be tied together by logic and temporal sequence, it is important to note that they can be executed independently or in different sequences – often, these experiments may not follow this logical path and different experiments will not be developed in a synchronous manner. For example, in case we already have information from our own studies or those from other labs indicating a specific brain region in a common behavioral assay, we may skip measuring from this brain region first and go straight to manipulating it. Or we use behavioral paradigms that are already established in our group and directly combine these with an intervention procedure based on pharmacogenetics. Or we may start out with component 5 to establish whether a certain assumed anatomical connection in the brain is capable of inducing molecular or physiological changes in another region of interest. Only after this functional connection is established, will a follow-up behavioral study be conducted.

It is important to note, that there is some overlap between the animal studies described in this project and those in earlier DEC-approved protocols. After a license for this project has been obtained, all experiments will formally be executed under this new license.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	**Establishing and characterizing rodent behavior that models compulsive behavior and its components**
2	**Identification of brain correlates of compulsive behavior and its components**
3	**Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation**
4	**Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation**
5	**Identification and characterization of neuroanatomical connections and their regulation**
6	
7	
8	
9	
10	

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101	
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.1	**Establishing and characterizing rodent behavior that models compulsive behavior and its component**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.1) is to answer the above questions 1 and 2:
A. to establish and validate animal models for compulsive behavior;
B. to characterize the tested population of animals (mice and rats) for individual differences;
C. to establish and validate behavioral methods to assess components of compulsive behavior, such as habit formation and cognitive flexibility;
D. to characterize the compulsivity in relation to its behavioral components.

The main outcome parameter of these procedures is behavior.
The behavioral procedures are meant to be used also in the subsequent appendices (3.4.4.2, 3.4.4.3 and 3.4.4.4). The results obtained form the basis to choose the best procedures and components to be used in combination with neuronal measurements and/or interventions. It is important to have these procedures validated and ready to use before the combination with invasive measurement and intervention methods, so that the behavioral procedures in animals with implants only will require some

fine-tuning.

Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

A. Introduce and validate models for compulsive behavior, so that they can be applied to answer our scientific questions.
We have chosen three rodent models as first tier models to start our studies. The choice was based on providing the best opportunity to discover a common neurobiological mechanism of the different compulsive phenotypes. The models are: Sapap3-mutant mice (compulsive grooming based on a genetic deletion of a postsynaptic density protein in the striatum); cocaine self-administration in rats (compulsive cocaine seeking and taking despite punishment); quinpirole-induced compulsive checking in rats (behavioral changes following repeated quinpirole administration). These are our first tier models, but they may be extended or replaced by other pharmacological, behavioral, genetic, and addiction models (via notification of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
The use of mice in addition to rats is based on the fact that transgenic mice such as the Sapap3-mutant mice are up to now the only model with spontaneous compulsive behavior, where no additional pharmacological treatment or behavioral training is required. The validation of the compulsive self-administration models (using cocaine or other drugs of abuse) depends on a test in which responding for the drug is punished. Compulsive animals will continue to respond despite the electrical foot shocks or substances that induce taste aversion (e.g., lithium chloride) that they receive.

B. Answer the scientific question how strong the individual differences are for the animal models of compulsivity that are validated under A.
Studying individual differences in behavior is an old approach that has proven its value over and over again. By studying the individual differences in compulsive behavior we aim to increase the chance that we will discover its underlying neurobiological mechanism. We already have experience with individual differences in two of our three first tier models, compulsive cocaine self administration (where only a limited number of rats will reach a level of compulsivity where they accept receiving foot-shocks when responding for cocaine) and Sapap3-mutant mice (where we find a high variability in the time spent grooming and where only intensely grooming mice respond to deep brain stimulation with a decrease in grooming).
We will follow this up by behaviorally testing animals from different strains and from lines with different genetic modifications. Initially, we will use two rat strains (our standard strain, Long Evans and another, to be selected on an expected clear difference in behavior) and two transgenic mouse strains (i.e. Sapap3-mutants and another, to be selected on an expected clear difference in behavior from our standard controls, C57Bl/6).
Another factor related to this is gender. For studies where animals are bred in our lab, we intend to use both males and females and this will provide us with the opportunity to collect important information on sex differences in compulsivity and the relation to underlying neurobiological mechanisms between sexes (see also section B. "The animals").

C. Introduction and validation of the "component" paradigms, so that they can be combined with the models for compulsive behavior and applied to answer our scientific questions.
The composition of compulsive behavior can be described as separate components: 1) persistence of performance, 2) elicitation of undesirable consequences, 3) escalation of symptoms over time, 4) aggravation by stress and anxiety, 5) exaggerated habit formation, 6) response inflexibility, and 7) loss of voluntary control. Whereas 1), 2), 3) and 7) are components that cannot be studied separately from the compulsive phenotype itself, 4), 5) and 6) may exist in the normal population and lower the threshold for compulsive behavior to develop. Our first interest is in the possible contribution of exaggerated habit formation and the relation of compulsivity with anxiety, but, dependent on the results obtained, the progress in the field and on the nature of the compulsivity models, we may later decide to focus on cognitive flexibility and the interaction with stress, as well. In the latter component we include the positive counterpart, i.e. possible alleviation of compulsivity by providing environmental enrichment.
Methods for fear learning may be established and validated when results with the other tests or new publications or hypotheses would make this test valuable to combine with models for compulsivity.
The cognitive flexibility component incorporates (through its training stages) also operant reward learning and decision-making. The habit test also based on operant reward responding, which, dependent

on the training schedules, leads to goal-directed or habit behavior. This is validated by using an outcome devaluation test, where the reward (outcome) is made less valuable for the animals, through pre-feeding or through association with sickness, induced by lithium chloride.
Anxiety is tested in an acute procedure, through exposure of the animal to an elevated-plus maze or an open field. Fear learning is tested in an associative procedure where the animal learns that a cue signals a foot-shock.
As these procedures need to be used in both rats and mice, they will be developed in validated in both rats and mice.
Interaction with stress generally will involve chronic exposure to various stressors (e.g. social defeat, restraint, forced swimming, foot-shocks, corticosterone administration). The positive counterpart of stress (environmental enrichment) involves social housing in regularly changing environments, e.g. large cages with many different components that are frequently altered, moved etc. We will use this type of enrichment to reverse or protect from compulsive behavior. Acute exposure to stress may also interact with compulsive behavior (only a single exposure) and will thus be implemented as well.

As it will need to be established what the best order of testing is for the combined experiments under D), in the procedures under C) we test the different options (see further under D)).

D. Answer what the relation is between compulsive behavior (in one of the models described under A and B) and separate components of that behavior (as described under C).
The three first tier models of compulsivity will be used while before or after the actual measurement of compulsive behavior the component behaviors are assessed.
Different orders of experimental tests may apply for different combinations. Thus, compulsivity testing in Sapap3-mutant mice involves the recording of spontaneous behavior. In the developmental course of the mice this may be repeated with regular intervals, so that the development of the compulsive grooming is followed. The anxiety test can also be repeated, so that over a long time course a possible relation between the two can be studied. In case of interaction with chronic stress or enrichment, compulsive grooming may be recorded both before and after the stress/enrichment exposure. Operant tests in the Sapap3-mutant mouse should preferably be performed at relatively young age, as responding is affected when the mice grow older and have a higher chance of developing lesions.
The training procedure for rats to reach a stage of compulsive cocaine self-administration is long. While anxiety testing can easily be combined with this, the other procedures require extended training periods as well, either for chronic exposure (stress, enrichment) or for (habit or flexibility) training. To keep experiments at a reasonable duration, we need to establish under C) in what order to apply a combination of tests/treatments. Similarly, establishment of experimental order apply to the combination of quinpirole treatment and the operant (habit/flexibility) tests.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

First tier **models** of compulsivity.
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks. Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-

3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

Second tier **models** of compulsivity.
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

Components of compulsivity.
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired

and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)
- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the

experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, animals with catheters, intracranial virus injections, repeated quinpirole treatments, chronic stress exposure and all Sapap3-mutant mice will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology.

A small subset of these animals (up to 25% but likely much less) may be used for the terminal experiments under anesthesia (3.4.4.5), for measurement of neuronal activity following acute intervention with brain activity. A small subset of these animals that were exclusively tested for rewarded behavior may be available for use in other experiments, as well.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Pilot experiments: establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on pilot studies and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species used:

Mice (mus musculus): genetically modified and wild type; mice are obtained from our own breedings or from a commercial licensed breeder.

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Behavioral studies contain an average of 10 rats or mice for each experimental group and control groups. Individual differences will be tested in 20 animals. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour) and accounting for a range of second tier models of compulsivity we will use 1500 animals in this appendix, 500 mice and 1000 rats. Of these, approx 30% will be exposed to mild and 70% to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.
We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in house. This will lead to a reduction of "breeding surplus".

Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4). However, a certain number (approx. up to 25%) of the animals in 3.4.4.1 will be transferred to one of the other procedures, most of them in 3.4.4.5 (terminal experiments under anesthesia).

Experiments will be executed in succession and, if needed, small explorative studies (pilots) will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in treatment-naïve animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the

maximum number of animals needed to obtain interpretable data.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Plasma sampling in animals for the measurement of cocaine or corticosterone concentrations in rats or mice, will be within in recommended/allowed limits.

Procedures will only be performed by competent personnel, as mandatory.
Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless food-restricted or implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment (see also F.). Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

In a subset of cases, such as after implantation of optic fibers, intravenous catheters etc., animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary.

In some cases, food restriction needs to be combined with isolated housing, when socially housed animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and

treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used).

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.
7. Chronic stress-exposed animals with catheters for plasma sampling need to handled leading to repeated mild discomfort.
8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Other aspects that may compromise the welfare of the animals are:
    - Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anesthetic, or accidental severing of nerve fibers or blood vessels.
    - Inflammation in the tissue around implanted devices such as intravenous catheters.
    - During intravenous drug self-administration animals sometimes overdose.
10. All animals will be frequently monitored for possible side effects. Animals exhibiting an unexpected phenotype with discomfort will be sacrificed immediately.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 8 is inherent to surgical procedures.

Surgical procedures cannot be executed with 0% failure rate and very seldom increased postoperative bleeding leads to maximally moderate discomfort.
There is considerable variability within rodent populations regarding the sensitivity to anaesthetics and drugs.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.

If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

We will use 1500 animals in this appendix, 500 mice and 1000 rats. Of these, approx 30% will be exposed to mild and 70% to moderate discomfort.

Of the compulsivity models, Sapap3-mutants may develop lesions. We estimate that in the course of the experiments 75% will experience maximally mild discomfort and 25% max moderate. If we expect that the discomfort will further increase, the animals are euthanized.

The addiction models all undergo surgery, may experience abstinence and foot-shocks when compulsivity is validated, together leading to maximally moderate discomfort.

The optogenetic stimulation models also undergo surgery, leading to max moderate discomfort.

Quinpirole and other pharmacological models may experience transient moderate discomfort.

During fear conditioning and chronic stress, animals may also experience moderate discomfort.
All other animals (including most control groups) wil experience no more than mild discomfort (food restriction plus behavioral testing).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Some animals (up to 25%) that were used for rewarded behavior in this appendix are available for use in other experiments and will not be sacrificed under this appendix (the majority of these 25% percent will be used for the terminal experiments under appendix 3.4.4.5).

All other animals will receive an overdose of Nembutal and perfused for brain fixation, immunohistochemistry and histology.

Subsequently, protein expression following virus injections, localization of implanted fibers and possible brain pathology in compulsivity models will be assessed.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 Type of animal procedure: **Identification of brain correlates of compulsive behavior and its components**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.2) is to answer the above question 3:
- to measure brain activity in behavioral paradigms for compulsive behavior and its components (such as habit formation or cognitive flexibility) in order to unravel the neurobiological underpinnings of this behavior.

The main outcome of these procedures in neuronal activity, in combination with behavior.
Thus, in these procedures behavioral tests described under 3.4.4.1 will be combined with one of the following "neuro-measurement" techniques to measure brain activity in awake rodents in our lab:
Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity
Measure-5) functional magnetic resonance imaging (fMRI) to assess whole-brain activity.

A maximum of two "neuro-measurement" techniques will be used in a single animal. In the vast majority

of cases only a single measurement technique is used in a single animal.
Both neuronal activity and neurotransmitter release are studied and measurements focus on both local and global processes. We need such an array of measurement techniques to increase the chance that we can identify the neurobiological correlates of the behavior studied and thus find targets for subsequent (3.4.4.3) intervention experiments.
Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

The general organization is:
A) to establish and validate the measurement techniques in animal models for compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory paradigm is obtained; that paradigm will be used (in 3.4.4.1).
B) to measure the neuronal activity parameter in the animal models – will deliver data of neuronal activity during compulsive behavior.
C) to establish and validate the measurement techniques when components of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory paradigm is obtained; that paradigm will be used.
D) to measure the neuronal activity parameter in the animal models while they are engaged in one of the components – will deliver data of neuronal activity during habit formation etc. in compulsive animals.

All neuro-measurement techniques (3.4.2.2) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement. Measure-1 to 4 require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays. Measure-5 (and in some cases Measure-4, when needed, so also D) requires head re-straining because movement artefacts will otherwise prevent measurements. The use of behavioral tests in combination with 4) is restricted and will mainly concern inducing a compulsive phenotype before measurements of possible alterations of resting state activity with fMRI. Pilot experiments (C) to combine conditioning tests (both appetitive and aversive) with fMRI measurements will be initiated and should lead to formal experiments under D.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Neuro-measurements are carried out in animal models of compulsivity and their controls. We'll start with our three first tier models (Sapap3-mutant mice, quinpirole-treated rats and cocaine self-administering rats) and combine these with 3 measurement techniques (electrophysiology, fast-scan cyclic voltammetry and fMRI). Electrophysiology and voltammetry will be used in all 3 models, but fMRI will be restricted to rats in the cocaine self-administration and quinpirole models.

When such measurements are combined with components of compulsivity the standard is to test anxiety and one other component (i.e., either habit formation, cognitive flexibility, sensitivity to stress, or fear conditioning). We'll first focus on habit formation, later on cognitive flexibility.

This procedure can consist of the following steps:

1. All animals (wild type or genetically manipulated) are housed together in single-sex groups until they become at least young adults (8 weeks of age). Two weeks before the start of behavioral experiments animals will be handled and weighed frequently.
2. Measurement equipment is implanted into the animals' brains through holes that are drilled into the skull (under adequate anesthesia and analgesia). In case of calcium imaging (Measure-4), a virus that will express Ca-indicating proteins, is infused (under adequate anesthesia and analgesia; at least 3-4 weeks recovery from this surgery to allow the virus to express). Animals will recover from anesthesia for at least one week.
3. Measurements in a model for compulsive behavior (group B) or compulsive behavior *and* its components (group D). Pilot experiments in groups A and C are used to establish the optimal sequence and timing of events.

Procedures Measure-1 to 4 (electrophysiology, voltammetry, microdialysis, Ca-imaging):
After the animals are connected to the recording/measuring set-up, they can move freely in the test box or test maze. The total time in the test will vary between 2-9 h. Daily electrophysiology and voltammetry

measurements can continue for up to 3 months. Microdialysis measurements can be repeated once. Ca-imaging can be repeated. Pilot experiments will be needed to establish the frequency and maximum number of measurements – a task that will involve frequent consultation of the IvD.

Procedures Measure-4 and Measure-5 (Ca- and fMRI-imaging):
Scanning animals in a MRI scanner (and in some cases calcium imaging (when using chronically implanted imaging windows)) requires head restraining to minimize head-movement-induced artefacts in the measurements. We follow a training protocol of 5 consecutive days with a duration of up to one hour each which reduces stress responses (corticosterone levels and observed restrained behavior). Non-coping animals will be removed from the experiment. After restraint training sessions concluded, animals will be transported to the MRI scanner (or calcium imaging apparatus). There the animals will be placed inside the scanner bore in our restrainer device, which has room for a custom build head coil specifically designed for rodents (and room for connecting the calcium imaging equipment).
Training duration: 1-2 weeks. Both Ca- and fMRI imaging may be repeated. Pilot experiments over the course of several months will be needed to establish the frequency and maximum number of measurements.

Sequence of experiments. Most of the models of compulsivity and also most of the components need acquisition/treatment periods of several weeks. The optimal sequence of events (compulsivity acquisition, intracranial implantation and measurements, acquisition of components) may vary: in group B the sequence may be implantation, measurement during compulsivity acquisition and expression; in group D: compulsivity acquisition, implantation, measurement during component acquisition (habit, flexibility, fear conditioning) or, alternatively, component (chronic stress or enrichment), implantation, compulsivity acquisition. The most suitable sequence (in terms of measurement success and animal discomfort) will be selected in pilot experiments in C).

*First tier **models** of compulsivity (**text identical to 3.4.4.1 is indicated in italics**).*
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks. Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final

phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

Second tier ***models*** *of compulsivity.*
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

Components *of compulsivity.*
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily

exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)
- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, animals with catheters, intracranial virus injections, repeated quinpirole treatments, chronic stress exposure and all Sapap3-mutant mice will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology.

Some of these animals (up to 25%) may be used for the terminal experiments under anesthesia (3.4.4.5), for measurement of neuronal activity following acute intervention with brain activity. A subset of these animals that were exclusively tested for behavior may be available for use in other experiments, as well. As all animals are implanted with electrodes, equipped with head posts etc. and have been through long behavioral procedures, they are not available for re-use in any protocol involving behavior.

After completion of the collection of data, the animals will be sacrificed (overdose of Nembutal and perfused for brain fixation) and their brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes, or stains to assess the effects of electrode stimulation). In the case of electrophysiology and voltammetry a small electrolytic lesion under proper isoflurane anesthesia will precede the Nembutal treatment and perfusion.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Pilot experiments: Establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species used:

Mice (mus musculus): genetically modified and wild type; mice are obtained from our own breedings or from a commercial licensed breeder.

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be

determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuromeasurement studies (3.4.4.2) contain an average of 20 animals (experimental group plus controls) plus 2 extra rats or mice for each experimental group and control groups, compared to the purely behavioral experiments of 3.4.4.1. This is to account for drop-out because of mis-placement and/or technical problems over the course of the experiments. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour; on average measurements and interventions are taking place on no more than a third of the overall experimental training days) we will use 1050 animals in this appendix, 350 mice and 700 rats. All (100%) will be exposed to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.
We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals. The basic testing of these intervention- and measurement-techniques will be performed as much as possible prior to performing an animal experiment.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in house, this will lead to a reduction of "breeding surplus".

Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4).

The measurement techniques that will be most frequently used (electrophysiology and fast-scan cyclic voltammetry) have been developed to allow chronic recordings in each animal. Thus, we will strive to perform experiments where each animal is his/her own control if possible (e.g. stimulation on vs stimulation off – this is also the way in which the clinical experiments are performed). In general, this also increases power and decreases the number of animals required.

Ca-imaging will be carried out using fiber implants and the animals can move around freely during recordings. The use of imaging windows which requires head fixation (and head fixation training) will be

avoided as much as possible.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in control animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the minimum number of animals needed to obtain interpretable data.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment. Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

In a subset of cases, such as after implantation of optic fibers, intravenous catheters etc., animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary.

In some cases, food restriction needs to be combined with isolated housing, when socially housed animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.
7. Chronic stress-exposed animals with catheters for plasma sampling need to handled leading to repeated mild discomfort.
8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Handling animals to connect implanted electrodes etc. to measurement equipment and, following behavioral and measurement sessions, disconnect them leads to repeated mild discomfort.
10. Rats used for fMRI measurements will undergo restraint training, that will not exceed moderate discomfort. Rats showing signs of non-coping will be taken out of the experiment.
11. Other aspects that may compromise the welfare of the animals are:

- Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anaesthetic, or accidental severing of nerve fibers or blood vessels.
- Inflammation in the tissue around implanted devices such as intravenous catheters.
- During intravenous drug self-administration animals sometimes overdose.
- Damage or loss of the head-stage/connector on the skull may lead to moderate discomfort. Animals will be taken out of the experiments when this happens.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 7 is inherent to surgical procedures.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anesthetics and drugs.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.
If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

Rats will be extensively handled and carefully trained for fMRI measurements. Rats that do not cope with the restraining training, will be taken out of the experiment. The restraining itself will be carried out under transient, light isoflurane anesthesia.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Level of discomfort: Neuro-measurement studies (as described here in 3.4.4.2 (and the behavioral aspect in 3.4.4.1)) last up to 3 months; up to 6 months when two are combined. In the majority of

paradigms, we food-deprive the animals (mild discomfort). Exceptions are drug self-adminstration studies (also mild discomfort due to drug withdrawal and catheter implantation) and studies only looking at measures of anxiety (mild discomfort due to experiencing fear and anxiety; or pain due to foot shocks) and spontaneous behavior (no discomfort (if not implanted with a headcap)). Of the SAPAP3 mutant mice, up to 50% will experience mild discomfort due to small skin lesions inflicted by excessive grooming (phenotype); the other 50% will be used before this phenotype develops. In addition, most animals will receive head implants or intracranial injections during a stereotaxic surgery for the measurement of brain activity. The recovery of this surgery is deemed moderate discomfort (for one week). Following recovery, wearing a cement headcap and being tethered to a commutator frequently will induce mild discomfort. Thus, we estimate 100% of the animals to experience mild discomfort throughout the experiments, with a period of moderate discomfort for up to one week after stereoxic surgeries. A small percentage of rats (up to 10% will undergo head restraining several times, which induces moderate discomfort.

In total, we estimate that of the 350 mice, 350 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereotactic surgeries or during training of head restraining.

Of the 700 rats, 700 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereotactic surgeries or during head restraining

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals will receive an overdose of Nembutal and perfused for brain fixation, immunohistochemistry and histology.

Subsequently, protein expression following virus injections, localization of implanted fibers and possible brain pathology in compulsivity models will be assessed.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101	
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.3	**Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.3) is to answer the above question 4 and 5:
- to manipulate brain activity in behavioral paradigms for compulsive behavior and its components.

The main outcome of these procedures is behavior.
Once a behavioral strategy is established and the desired behavior is detected, brain activity during this behavior is manipulated in order to unravel the neurobiological underpinnings of this behavior. To discover causal relationships between brain activity and behavior, behavior will be measured while brain activity is manipulated using the following interventions:

The aim of these procedures is
Intervent-1) Deep-brain stimulation (DBS)
Intervent-2) pharmacogenetics
Intervent-3) optogenetics

Intervent-4) lesions
Intervent-5) pharmacological treatments

A maximum of two "neuro-intervention" techniques will be used in a single animal. In the vast majority of cases only a single intervention technique is used in a single animal.

These techniques allow interventions of both local and global processes, with different levels of spatial, cellular and pharmacological selectivity. We need such an array of intervention techniques to increase the chance that we can identify the neurobiological underpinnings of the behavior studied and find ways to alter compulsive behavior.
Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

The general organization is:
A) to establish and validate the intervention techniques in animal models for compulsive behavior – in pilot experiments a satisfactory paradigm is selected for further use (in 3.4.4.1);.
B) to alter the compulsive behavior in the animal models –will deliver data of efficacy potential of target structures and/or cellular processes.
C) to establish and validate the intervention techniques when components of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments a satisfactory paradigm is selected for further studies.
D) to alter behavior in the animal models while they are engaged in one of the components – will deliver efficacy potential data of target structures and/or cellular processes.

All neuro-intervention techniques will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement (Intervent-1,-2,-3, and -5). Exceptions are Intervent-4, where a one-off local microinjection is performed. In Intervent-2 and Intervent-5 pharmacological agents can be administered peripherally or centrally through the implanted cannula.
Intervent-1 and Intervent-3 involve continuous application of electrical or light pulses and therefore require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Neuro-interventions are carried out in animal models of compulsivity and their controls. We'll start with our three first tier models (Sapap3-mutant mice, quinpirole-treated rats and cocaine self-administering rats) and combine these with 3 intervention techniques (deep brain stimulation, optogenetics, pharmacogenetics).

This procedure can consist of the following steps (including steps described in 3.4.4.1, see below):
1. Animals are housed together until they become at least young adults (6-8 weeks of age). Then they are handled and weighed every week.
2. In case of pharmacogenetics (Intervent-2) and optogenetics (Intervent-3), viruses that will express proteins that will make infected neurons sensitive to pharmacological or optical treatment, are infused intracranially. If possible, in the same surgical session, intervention devices are implanted into the animals' brains through holes that are drilled into the skull (under adequate anaesthesia and analgesia). Depending on the technique, the equipment consists of electrodes (Intervent-1), a guide cannula to enable the infusion of pharmacological agents (Intervent-2,-4, and -5), or fiber optics (Intervent-3).
3. Animals will acutely recover from anaesthesia in their home cages on a heating plate. Subsequently, long-term recovery from surgery will last one week, in which the animals will not undergo any additional experimental procedures causing discomfort.
4. Training in a model for compulsive behavior and/or its components.
5. Application of "Neuro-intervention" techniques to alter behavior in awake rodents behaving in paradigms listed below.

Procedures Intervent-1 and -3:
After transport from the housing room to the experimental room, animals will be habituated to the room

for one hour. Then animals will be introduced to the testing environment and connected to the intervention set-up. In some cases, the head-implanted equipment (if present) is connected to the recording set-up by a cable that runs through an optical (C) or electrical (A) commutator (swivel) mounted above them, allowing free movement in the experimental cage. In other cases, a commutator is not required (D and E) or a wireless device is used (A). After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be disconnected, removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for connecting and disconnecting the animals]. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to maximally 3 months in some cases]
At the end of the experiment, the animals will be given an overdose of Nembutal and perfused for brain fixation, histology, and immunohistochemistry. In case of DBS (A), a weak current is applied prior to perfusion to the stimulating electrode for up to 30 seconds to mark the position of the electrodes with a small electrolytic lesion under proper isoflurane anaesthesia (no discomfort for the animal).

Procedures Intervent-2 and -5:
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. On some days, animals will be infused intracranially with pharmacological agents by introducing an infusion cannula into the previously implanted guide cannula. Subsequently, the animals are introduced to the testing environment. After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for infusing the animals and letting the intervention drug act]. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to maximally 3 months in some cases]
At the end of the experiment, the animals will be given an overdose of Nembutal and perfused for brain fixation, histology, and immunohistochemistry.

Procedure Intervent-4:
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. Subsequently, the animals are introduced to the testing environment. After behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately test 1-6 hours. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to maximally 3 months in some cases]
At the end of the experiment, the animals will be given an overdose of Nembutal and perfused for brain fixation, histology, and immunohistochemistry.

Sequence of experiments. Most of the models of compulsivity and also most of the components need acquisition/treatment periods of several weeks. The optimal sequence of events may vary: in some cases the sequence may be implantation, measurement during compulsivity acquisition and expression; in other cases the sequence compulsivity acquisition, implantation, measurement during component acquisition (habit, flexibility, fear conditioning) might be preferred or, alternatively, component (chronic stress or enrichment), implantation, compulsivity acquisition. The most suitable sequence (in terms of measurement success and animal discomfort) will be selected in pilot experiments in C).

*First tier **models** of compulsivity (**text identical to 3.4.4.1 is indicated in italics**).*
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks.

Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

*Second tier **models** of compulsivity.*
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

***Components** of compulsivity.*
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a

Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)

- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, all animals will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology. The brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes or cannulae, or stains to assess the effects of electrode stimulation). In the case of virus injections the expression of the light- or drug-sensitive proteins will be visualized.

As all animals are implanted with electrodes, equipped with head posts etc. and have been through long behavioral procedures, they are not available for re-use in any protocol involving behavior.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Pilot experiments: Establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant – all of which will be communicated to and checked by the IvD.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species used:

Mice (mus musculus): genetically modified and wild type; mice are obtained from our own breedings or from a commercial licensed breeder.

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-

house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuro-intervention studies (3.4.4.3) contain an average of 20 animals (experimental group plus controls) plus 2 extra rats or mice for each experimental group and control groups, compared to the purely behavioral experiments of 3.4.4.1. This is to account for drop-out because of mis-placement and/or technical problems over the course of the experiments. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour; on average measurements and interventions are taking place on no more than a third of the overall experimental training days) we will use approximately 1050 animals in this appendix, 350 mice and 700 rats. All (100%) will be exposed to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.
We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals. The basic testing of these intervention- and measurement-techniques will be performed as much as possible prior to performing an animal experiment.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.
We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in

house, this will lead to a reduction of "breeding surplus".

Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4).

The intervention techniques that will be most frequently used (deep brain stimulation and optogenetics) allow repeated interventions and interventions using a wide range of different parameters in each animal. Thus, we will strive to perform experiments where each animal is his/her own control if possible (e.g. stimulation on vs stimulation off – stimulation A vs stimulation B etc; this is also the way in which the clinical experiments are performed). In general, this also increases power and decreases the number of animals required.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in control animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the maximum number of animals needed to obtain interpretable data.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anaesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment. Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

In most cases, such as after implantation of optic fibers, intravenous catheters etc., animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary. In some cases, food restriction needs to be combined with isolated housing, when socially housed

animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used).

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, Increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.

7. Chronic stress-exposed animals with catheters for plasma sampling need to be handled leading to repeated mild discomfort.
8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Handling animals to connect implanted electrodes etc to measurement equipment and, following behavioral and measurement sessions, disconnect them leads to repeated mild discomfort.
10. Neuro-intervention is used to induce behavioral changes. While the aim is to reduce compulsive (pathological) behavior, it cannot be completely avoided that we may also sometimes induce unwanted behavior. In the pilot experiments we will carefully select the parameters for DBS and optogenetic stimulation, the targets for lesions, local microinfusions of pharmacological agents so that no extra discomfort is caused when the formal experiments are performed.
11. Other aspects that may compromise the welfare of the animals are:
    - Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anaesthetic, or accidental severing of nerve fibers or blood vessels.
    - Inflammation in the tissue around implanted devices such as intravenous catheters.
    - During intravenous drug self-administration animals sometimes overdose.

Damage or loss of the head-stage/connector on the skull may lead to moderate discomfort. Animals will be taken out of the experiments when this happens.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 and 9 and 10 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 8 is inherent to surgical procedures.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and very seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anaesthetics and drugs.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.

If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older

(> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Level of discomfort: Neuro-intervention studies (as described here in 3.4.4.3 (and the behavioral aspect in 3.4.4.1)) last up to 3 months; up to 6 months when two are combined. In the majority of paradigms, we food-deprive the animals (mild discomfort). Exceptions are drug self-adminstration studies (also mild discomfort due to drug withdrawal and catheter implantation) and studies only looking at measures of anxiety (mild discomfort due to experiencing fear and anxiety; or pain due to foot shocks) and spontaneous behavior (no discomfort (if not implanted with a headcap)). Of the SAPAP3 mutant mice, up to 50% will experience mild discomfort due to small skin lesions inflicted by excessive grooming (phenotype); the other 50% will be used before this phenotype develops. In addition, most animals will receive head implants or intracranial injections during a stereotaxic surgery for the measurement of brain activity. The recovery of this surgery is deemed moderate discomfort (for one week). Following recovery, wearing a cement headcap and being tethered to a commutator frequently will induce mild discomfort. Thus, we estimate 100% of the animals to experience mild discomfort throughout the experiments, with a period of moderate discomfort for up to one week after stereoxic surgeries. A small percentage of rats (up to 10% will undergo head restraining several times, which induces moderate discomfort.

In total, we estimate that of the 350 mice, 350 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereotactic surgeries or during training of head restraining (→ cumulative moderate).

Of the 700 rats, 700 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after steretactic surgeries or during head restraining (→ cumulative moderate).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

All animals will receive an overdose of Nembutal and perfused for brain fixation, immunohistochemistry and histology.

Subsequently, protein expression following virus injections, localization of implanted fibers and possible brain pathology in compulsivity models will be assessed.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101	
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.4	**Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.4) is to answer the above questions 3, 4, and 5:
- to identify neuroanatomical connections between brain regions involved in compulsive behavior (and its components) and to characterize how these brain regions interact with and regulate each other.

Neuro-intervention and neuro-measurement in awake behaving animals (only reached in a relatively small number of animals): Brain activity will be manipulated (excitation or inhibition) at the neuronal or network level using pharmacology, optogenetics, pharmacogenetics, deep-brain stimulation (DBS) or by performing lesions (see 3.4.4.2). These techniques can facilitate or disrupt the activity of a group of neurons in a local region (e.g., optogenetics), neurotransmitter systems or entire brain networks (e.g., DBS). Such interventions will allow us to establish causal relationships between behavior and neural correlates of interest, which is one of the key aims of this proposal. For these experiments, we will measure the difference in neuronal responses and behavior (simultaneously) between a baseline

time when the manipulation had not been performed and following this intervention. Measurements will be collected using neurobiological activity using calcium imaging, electrophysiology, electrochemistry, microdialysis, and fMRI (also see 3.4.4.3).

The main outcome parameter of these procedures is neuronal activity, in combination with behavior.
Thus, in these procedures "neuro-measurement" and "neuro-intervention" techniques described under 3.4.4.2 and 3.4.4.3, respectively, will be combined to study brain activity and interaction between brain systems in the awake behaving animal. Thus, in these procedures behavioral tests described under 3.4.4.1 will be combined with one of the following "neuro-measurement" techniques to measure brain activity in awake rodents in our lab (techniques previously described in **3.4.4.2**):

Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity
Measure-5) functional magnetic resonance imaging (fMRI) to assess whole-brain activity.

"Neuro-intervention" techniques to measure brain activity in awake rodents in our lab (techniques previously described in **3.4.4.3**) are:
Intervent-1) Deep-brain stimulation (DBS)
Intervent-2) pharmacogenetics
Intervent-3) optogenetics
Intervent-4) lesions
Intervent-5) pharmacological treatments

One "neuro-measurement" technique will be combined with one "neuro-intervention" technique.

Both neuronal activity and neurotransmitter release are studied and measurements focus on both local and global processes. We need such an array of measurement and intervention techniques to increase the chance that we can identify the neurobiological correlates of the behavior studied and thus find targets for subsequent (3.4.4.3) intervention experiments.
Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

The general organization is:
A) to establish and validate the combination of measurement and intervention techniques in animal models for compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory solution is obtained.
B) to measure the neuronal activity parameter in the behaving individuals of animal models for compulsive behavior in response to neuro-intervention – will deliver data of neuronal activity during compulsive behavior.
C) to establish and validate the combination of measurement and intervention techniques when components of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory solution is obtained.
D) to measure and manipulate neuronal activity parameters in the animal models and controls while they are engaged in one of the components – will deliver data of neuronal activity during habit formation etc. in compulsive animals.

All neuro-measurement techniques (3.4.4.2) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement. Techniques Measurement-1 to -4 require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays.
Measurement-5 (and in some cases measurement-4) requires head re-straining because movement artefacts will otherwise prevent measurements. Pilot experiments (C) to combine conditioning tests (both appetitive and aversive) with fMRI measurements will be initiated and should lead to formal experiments under D.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Neuro-measurements and neuro-interventions are carried out in animal models of compulsivity and their controls. We'll start with our three first tier models (Sapap3-mutant mice, quinpirole-treated rats and cocaine self-administering rats) and combine these with 3 measurement techniques (electrophysiology, fast-scan cyclic voltammetry and fMRI) and a selection of intervention techniques (see above). Electrophysiology and voltammetry will be used in all 3 models, but fMRI will be restricted to rats in the cocaine self-administration and quinpirole models.
When such measurements are combined with components of compulsivity the standard is to test anxiety and one other component (i.e., either habit formation, cognitive flexibility, sensitivity to stress, or fear conditioning). We'll first focus on habit formation, later on cognitive flexibility.
This procedure can consist of the following steps:

1. All animals (wildtype or genetically manipulated) are housed together in single-sex groups until they become at least young adults (8 weeks of age). Two weeks before the start of behavioral experiments animals will be handled and weighed frequently.
2. Measurement and intervention equipment is implanted into the animals' brains through holes that are drilled into the skull (under adequate anesthesia and analgesia). In case of calcium imaging (measurement-4), a virus that will express Ca-indicating proteins, is infused (under adequate anesthesia and analgesia; at least 3-4 weeks recovery from this surgery to allow the virus to express). Animals will recover from anesthesia for at least one week.
   Depending on the technique, the equipment consists of electrodes (Measreument-1 and measurement-2), a guide cannula to enable lowering of electrodes (measurement-2) or a semipermeable membrane (measurement-3), fiber optics (measurement-4), or a post for head fixation (measurement-4 and measurement-5). In case of calcium imaging (measurement-4), a virus that will express proteins that make calcium fluorescent and thus optically detectable, is infused.
   During the same surgery, intervention devices are implanted into the animals' brains. Depending on the technique, the equipment consists of electrodes (intervention-1), a guide cannula to enable the infusion of pharmacological agents (intervention-2, intervention-4, and intervention-5), or fiber optics (intervention-3). In case of pharmacogenetics (intervention-2) and optogenetics (intervention-3), a virus that will express proteins that will make infected neurons sensitive to pharmacological (e.g., clozapine-N-oxide) or optical (e.g., light manipulating so-called opsins) treatment, is infused.
3. Measurements and interventions in a model for compulsive behavior (group B) or compulsive behavior *and* its components (group D). Pilot experiments in groups A and C are used to establish the optimal sequence and timing of events.

The measurement and intervention procedures can consist of the following steps (including steps described in 3.4.4.1, see below):

Measurement-1 to -4 (electrophysiology, voltammetry, microdialysis, Ca-imaging):
After the animals are connected to the recording/measuring set-up, they can move freely in the test box or test maze. The total time in the test will vary between 2-9 h. Daily electrophysiology and voltammetry measurements can continue for up to 3 months. Microdialysis measurements can be repeated once. Ca-imaging can be repeated. Pilot experiments will be needed to establish the frequency and maximum number of measurements.

Measurement-4 or -5 (Ca- & fMRI-imaging):
Scanning animals in a MRI scanner (and in some cases calcium imaging) requires head restraining to minimize head-movement-induced artefacts in the measurements. We follow a training protocol of 5 consecutive days with a duration of up to one hour each which reduces stress responses (corticosterone levels and observed restrained behavior). Non-coping animals will be removed from the experiment.
After restraint training sessions concluded, animals will be transported to the MRI scanner (or calcium imaging apparatus). There the animals will be placed inside the scanner bore in our restrainer device, which has room for a custom build head coil specifically designed for rodents (and room for connecting the calcium imaging equipment).
Training duration: 1-2 weeks. Both Ca- and fMRI imaging may be repeated. Pilot experiments will be needed to establish the frequency and maximum number of measurements.

Intervention-1 or -3 (DBS & optogenetics):
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. Then animals will be introduced to the testing environment and connected to the intervention set-up. In some cases, the head-implanted equipment (if present) is connected to the

recording set-up by a cable that runs through an optical (intervention-3) or electrical (intervention-1) commutator (swivel) mounted above them, allowing free movement in the experimental cage. In other cases, a commutator is not required (intervention-4 and -5) or a wireless device is used (intervention-1). After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be disconnected, removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for connecting and disconnecting the animals].

Intervention-2 or -5 (pharmacogenetics & pharmacological treatments):
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. On some days, animals will be infused intracranially with pharmacological agents by introducing an infusion cannula into the previously implanted guide cannula. Subsequently, the animals are introduced to the testing environment. After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for infusing the animals and letting the intervention drug act].

Intervention-4 (lesions):
After transport from the housing room to the experimental room, animals (with excitotoxic lesions of specific brain regions of interest) will be habituated to the room for one hour. Subsequently, the animals are introduced to the testing environment. After behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately test 1-6 hours. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to 3 months]

Sequence of experiments. Most of the models of compulsivity and also most of the components need acquisition/treatment periods of several weeks. The optimal sequence of events (compulsivity acquisition, intracranial implantation and measurements, acquisition of components) may vary: in group B the sequence may be implantation, measurement during compulsivity acquisition and expression; in group D: compulsivity acquisition, implantation, measurement during component acquisition (habit, flexibility, fear conditioning) or, alternatively, component (chronic stress or enrichment), implantation, compulsivity acquisition. The most suitable sequence (in terms of measurement success and animal discomfort) will be selected in pilot experiments in C).

*First tier **models** of compulsivity (**text identical to 3.4.4.1 is indicated in italics**).*
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks. Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their

free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

*Second tier **models** of compulsivity.*
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

***Components** of compulsivity.*
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)
- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, animals with catheters, intracranial virus injections, repeated quinpirole treatments, chronic stress exposure and all Sapap3-mutant mice will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology.

After completion of the collection of data, the animals will be sacrificed (overdose of Nembutal and perfused for brain fixation) and their brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes, or stains to assess the effects of electrode stimulation). In the case of electrophysiology and voltammetry a small electrolytic lesion under proper isoflurane anesthesia will precede the Nembutal treatment and perfusion.

As all animals are implanted with electrodes, equipped with head posts etc. and have been through long behavioral procedures, they are not available for re-use in any protocol involving behavior.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Pilot experiments: Establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species used:

Mice (mus musculus): genetically modified and wild type; mice are obtained from our own breedings or from a commercial licensed breeder.

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and

females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuro-intervention/measurement studies (3.4.4.4) contain an average of 20 animals (experimental group plus controls) plus 2 extra rats or mice for each experimental group and control groups, compared to the purely behavioral experiments of 3.4.4.1. This is to account for drop-out because of mis-placement and/or technical problems over the course of the experiments. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour; on average measurements and interventions are taking place on no more than a third of the overall experimental training days) we will use 300 animals in this appendix, 150 mice and 150 rats. All (100%) will be exposed to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.

We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals. The basic testing of these intervention- and measurement-techniques will be performed as much as possible prior to performing an animal experiment.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in house, this will lead to a reduction of "breeding surplus".
Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4).

The measurement techniques that will be most frequently used (electrophysiology and fast-scan cyclic

voltammetry) have been developed to allow chronic recordings in each animal. Thus, we will strive to perform experiments where each animal is his/her own control if possible (e.g. stimulation on vs stimulation off – this is also the way in which the clinical experiments are performed). In general, this also increases power and decreases the number of animals required.

Ca-imaging will be carried out using fiber implants and the use of imaging windows requiring head fixation (and head fixation training) will be avoided as much as possible.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in control animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the maximum number of animals needed to obtain interpretable data.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment. Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed experiments are fundamental research, an are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

☒ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

In all cases animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary.
In some cases, food restriction needs to be combined with isolated housing, when socially housed animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

Check the answer given in procedure 3.4.4.1.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☒ No > Justify why pain relieving methods will not be used.

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used).

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.
7. Chronic stress-exposed animals with catheters for plasma sampling need to be handled leading to repeated mild discomfort.

8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Handling animals to connect implanted electrodes etc. to measurement equipment and, following behavioral and measurement sessions, disconnect them leads to repeated mild discomfort.
10. Rats used for fMRI measurements will undergo restraint training, that will not exceed moderate discomfort. Rats showing signs of non-coping will be taken out of the experiment.
11. Other aspects that may compromise the welfare of the animals are:
    - Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anesthetic, or accidental severing of nerve fibers or blood vessels.
    - Inflammation in the tissue around implanted devices such as intravenous catheters.
    - During intravenous drug self-administration animals sometimes overdose.

Damage or loss of the head-stage/connector on the skull may lead to moderate discomfort. Animals will be taken out of the experiments when this happens.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 7 is inherent to surgical procedures.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anesthetics and drugs.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.

If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

Rats will be extensively handled and carefully trained for fMRI measurements. Rats that do not cope with the restraining training, will be taken out of the experiment. The restraining itself will be carried out under transient, light isoflurane anesthesia.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Level of discomfort: Neuro-intervention/measurement studies (as described here in 3.4.4.4 (and the behavioral aspect in 3.4.4.1)) last up to 3 months; up to 6 months when two are combined. In the majority of paradigms, we food-restrict the animals (mild discomfort). Exceptions are drug self-adminstration studies (also mild discomfort due to drug withdrawal and catheter implantation) and studies only looking at measures of anxiety (mild discomfort due to experiencing fear and anxiety; or pain due to foot shocks) and spontaneous behavior (no discomfort (if not implanted with a headcap)). Of the SAPAP3 mutant mice, up to 50% will experience mild discomfort due to small skin lesions inflicted by excessive grooming (phenotype); the other 50% will be used before this phenotype develops. In addition, most animals will receive head implants or intracranial injections during a stereotaxic surgery for the measurement of brain activity. The recovery of this surgery is deemed moderate discomfort (for one week). Following recovery, wearing a cement headcap and being tethered to a commutator frequently will induce mild discomfort. Thus, we estimate 100% of the animals to experience mild discomfort throughout the experiments, with a period of moderate discomfort for up to one week after stereoxic surgeries. A small percentage of rats (up to 10% will undergo head restraining several times, which induces moderate discomfort.

In total, we estimate that of the 150 mice, 150 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereoxic surgeries or during head restraining.

Of the 150 rats, 150 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereoxic surgeries or during head restraining.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Rats and mice will be killed for histological and immunohistochemical analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.5 Type of animal procedure: **Identification and characterization of neuroanatomical connections and their regulation**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.5) is to answer the above questions 3 and 5:
- to identify neuroanatomical connections between brain regions involved in compulsive behavior (and its components) and to characterize how these brain regions interact with and regulate each other.

The main outcome parameter of these procedures is neuronal activity (in the anesthetized animals and in brain slices).
Thus, in these procedures "neuro-measurement" and "neuro-intervention" techniques described under 3.4.4.2 and 3.4.4.3, respectively, will be combined to study brain activity and interaction between brain systems in anesthetized rodents or brain slices. Thus, this appendix is identical to 3.4.4.4 (also combines "neuro-measurement" and "neuro-intervention" techniques) except that it is carried out in an additional set of animals in the anesthetized preparation or in brain slices, so behavior is not taken into account. Furthermore, these experiments are conducted acutely, thus the procedures are non-survival. This acute approach enables the direct manipulation and measurement of brain circuits without the additional

complication of performing these techniques in behaving animals (needs no chronic implantation of tools into brain/skull and warrants less noisy recordings due to a better controlled environment and the absence of movement artefacts), and thus allows faster and more efficient testing of hypotheses regarding how brain regions of interest interact.

Neuro-intervention and neuro-measurement in anesthetized animals: Brain activity will be manipulated (excitation or inhibition) at the neuronal or network level using pharmacology, optogenetics, pharmacogenetics, deep-brain stimulation (DBS) or by performing lesions (see 3.4.4.2). These techniques can facilitate or disrupt the activity of a group of neurons in a local region (e.g., optogenetics), neurotransmitter systems or entire brain networks (e.g., DBS). Such interventions will allow us to establish causal relationships between neural correlates of interest, which is one of the key aims of this proposal. For these experiments, we will measure the difference in neuronal responses between a baseline time when the manipulation had not been performed and following this intervention. Measurements will be collected using neurobiological activity using calcium imaging, electrophysiology, electrochemistry, microdialysis, and fMRI (see 3.4.4.3).

"Neuro-measurement" techniques to measure brain activity in anesthetized rodents (techniques previously described in **3.4.4.2**) are:
Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity

Both neuronal activity and neurotransmitter release are studied and measurements focus on both local and global processes. We need such an array of measurement techniques to increase the chance that we can identify the neurobiological correlates of the behavior studied and thus find targets for subsequent intervention experiments (3.4.4.3), as well as targets to perform subsequent measurement experiments (3.4.4.2), or the combination of the two (3.4.4.4).

"Neuro-intervention" techniques to measure brain activity in anesthetized rodents (techniques previously described in **3.4.4.3**) are:
Intervent-1) Deep-brain stimulation (DBS)
Intervent-2) pharmacogenetics
Intervent-3) optogenetics
Intervent-4) lesions
Intervent-5) pharmacological treatments

One "neuro-measurement" technique will be combined with one "neuro-intervention" technique.

Neuro-intervention and neuro-measurement in brain slices: After sacrificing the animal and collecting the brain, neuronal activity will be manipulated in brain slices using pharmacology, optogenetics, pharmacogenetics, or by DBS (see 3.4.4.2). Such interventions will allow us to establish causal relationships between neural correlates of interest. Measurements will be collected using neurobiological activity using calcium imaging, electrophysiology, and electrochemistry (see 3.4.4.3).

In a subset of animals, we will perform this procedure (3.4.4.5) at the end of a pilot study/experiment carried out under a different procedure (up to 25% of the animals from 3.4.4.1; and potentially in a small set of animals from 3.4.4.2, 3.4.4.3, and 3.4.4.4) to either reduce the number of animals needed in our project or to assess the effects of previous experience on brain function. In either scenario, no discomfort would be added.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

This procedure can consist of the following steps:

1. Animals are housed together until they become at least young adults (8 weeks of age). Then they are handled and weighed frequently.
2. (optional) In case of calcium imaging (Measure-4), a virus (e.g., AAV) that will express proteins that make calcium fluorescent and thus optically detectable, is infused into the brain via stereotactic microinfusion (under proper anesthesia (e.g., isoflurane) and perioperative analgesia; at least 3-4 weeks recovery from this surgery to allow the virus to express). Similarly, in case of

pharmacogenetics (Intervent-2) and optogenetics (Intervent-3), a virus that will express proteins that will make infected neurons sensitive to pharmacological (e.g., clozapine-N-oxide) or optical (e.g., light-sensitive so-called opsins) treatment, is infused.

3. For the anesthetized experiments: Measurement devices are acutely lowered into the animals' brains through holes that are drilled into the skull (under proper anesthesia (e.g., urethane) and perioperative analgesia). Some the equipment will be anchored onto the animals' skull with screws and dental cement. Depending on the technique, the devices consist of electrodes (Measure-1 and Measure-2), a guide cannula to enable lowering of electrodes (Measure-2) or a semipermeable membrane (Measure-3), fiber optics (Measure-4), or a post for head fixation (Measure-4). During the same surgery, intervention devices are lowered into the animals' brains. Some of the devices will be anchored onto the animals' skull with screws and dental cement. Depending on the technique, the devices consists of electrodes (Intervent-1), a guide cannula to enable the infusion of pharmacological agents (Intervent-2, Intervent-4, and Intervent-5), or fiber optics (Intervent-3).
   For the brain slice experiments: Animals will be sacrificed and the above mentioned measurement and intervention techniques will be applied in brain slices.

Testing of females. When we use female animals, estrous cycle may be checked frequently to control for potential sex hormonal effects on the brain and to determine when to conduct crucial parts of the experiments (e.g., experiments on females should all take place in the same period of the estrous cycle to prevent divergent effects of sex hormones on the brain). A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

After completion of the collection of data, the animals will be sacrificed (overdose of Nembutal and perfused for brain fixation) and their brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes/other devices, stains to assess viral expression, and/or stains to assess the effects of "neuro-intervention" (e.g., electrode stimulation)). In the case of electrophysiology and voltammetry a small electrolytic lesion under continued anesthesia will precede the Nembutal treatment and perfusion (animal is still under proper anesthesia; no additional discomfort for the animal). Thus, animals will not recover from surgery/anesthesia.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Pilot experiments: Establishing new or adapted procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Species used:

Mice (mus musculus): genetically modified and wild type; mice are obtained from our own breedings or from a commercial licensed breeder.

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition,

measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuro-intervention/measurement studies in anesthetized animals (3.4.4.5) contain an average of 20 animals (experimental group plus controls). Based on the present plans, we will use 1100 animals in this appendix, 400 mice and 700 rats. Approximately 75% of the animals will be exposed to moderate discomfort (recovery from stereotactic surgery for injection of virus or pharmacological agents), and the remaining 25% will experience mild discomfort. Approximately 75% of the animals will be used in the anesthetized preparation, and the remaining 25% will be used for *in vitro* slice experiments (discomfort does not differ between the two because both procedures are non-survival).

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

These types of studies are conducted in both rats and mice worldwide, making translation and extrapolation of data between research-groups feasible.

Furthermore, the procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. On basis of this previous work and

experience, statistical analysis can be performed to determine the maximum number of animals needed to obtain interpretable data.

In principle we will use both male and female rats and mice. In particular in the case of the (transgenic) animals that are bred in house, this will lead to a reduction of "breeding surplus".
Some animals from 3.4.4.1 (up to 25% of 3.4.4.1 animals; but probably significantly fewer) and in small numbers from 3.4.4.2, 3.4.4.3, and 3.4.4.4 will be transferred to 3.4.4.5 for further non-behavioral experimentation to reduce the total number of animals used for our project.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia (and analgesia).

In case of stereotactic intracerebral injections of viruses or other agents (75% of the animals) prior to the experiment, close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed and provided with environmental enrichment.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

Adequate anaesthesia and analgesia is used for all procedures.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other aspects that may compromise the welfare of the animals are:

- In case of stereotactic intracerebral injections of viruses or other agents (75% of the animals) prior to the experiment, recovery from stereotactic surgery may lead to maximally moderate discomfort.

- Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anesthetic, or accidental severing of nerve fibers or blood vessels.

Explain why these effects may emerge.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anesthetics and drugs.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Level of discomfort: Most animals (~ 75%) will receive an intracranial injection during a stereotactic surgery prior to the above described non-survival procedure. The recovery of this surgery is deemed moderate discomfort (for one week). Thus, we estimate up to 75% of the animals to experience a period of moderate discomfort for up to one week after stereotactic surgeries; but not discomfort otherwise

because these experiments are non-survival.

In total, we estimate that of the 400 mice, 300 will experience a period of moderate discomfort for up to one week after stereotactic surgeries; the remaining 100 will experience mild discomfort.

Of the 700 rats, 525 will experience a period of moderate discomfort for up to one week after stereotactic surgeries; the remaining 175 will experience mild discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Rats and mice will be killed for histological and immunohistochemical analyses.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

principal approach

additional approach

3.4.4.1 establishing and characterizing rodent behavior that models compulsive behavior and its components
main read-out: **behavior**

models of compulsive behavior,

- induced by:
  - drug self-administration
  - genetic modification
  - pharmacological treatment
  - optogenetic stimulation
  - behavioral conditions
- characterized by:
  - escalation & persistence despite negative consequences

are tested for components:

- habit-formation
- cognitive flexibility
- fear and anxiety
- aggravation by stress , alleviation by environmental enrichment

3.4.4.2 identification of brain correlates of compulsive behavior and its components
main read-out: **behavior** & **neuronal activity**

behavioral testing (see box on the left)

plus neuro-measurement
1) electrophysiology
2) electrochemistry
3) microdialysis
4) calcium imaging
5) fMRI

3.4.4.3 establishing causality between brain pathways and compulsive behavior and its components via brain manipulation
main read-out: **behavior**

behavioral testing (see box on the left)

plus neuro-intervention
1) deep-brain stimulation
2) pharmacogenetics
3) optogenetics
4) lesions
5) pharmacology

3.4.4.4 establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation
main read-out: **behavior** & **neuronal activity**

behavioral testing (see box on the left)

plus neuro-measurement & neuro-intervention

1 measurement technique + 1 intervention technique

3.4.4.5 identification and characterization of neuroanatomical connections and their regulation
main read-out: **neuronal activity**

neuro-measurement & neuro-intervention in anesthetized animals 1 measurement technique + 1 intervention technique

Project [redacted] - AVD-801002015126 - Nederlands Herseninstituut-KNAW
Neurobiology of compulsive behavior and its components: Brain stimulation and measurements

	Procedure			mild	moderate
3.4.4.1	**Behavioral testing only**	Group 1a	rats	300	
		Group 1b	rats		700
		Group 1c	mice	150	
		Group 1d	mice		350
3.4.4.2	**Behavioral testing plus neuro-measurement**	Group 2a	rats		700
		Group 2b	mice		350
3.4.4.3	**Behavioral testing plus neuro-intervention**	Group 3a	rats		700
		Group 3b	mice		350
3.4.4.4	**Behavioral testing plus neuro-measurement and neuro-intervention**	Group 4a	rats		150
		Group 4b	mice		150
3.4.4.5	**Neuro-measurement and neuro-intervention in anesthetized animals**	Group 5a	rats	175	
		Group 5b	rats		525
		Group 5c	mice	100	
		Group 5d	mice		300
Total rat		**3250**		475 14.6%	2775 85.4%
Total mice		**1750**		250 14.3%	1500 85.7%

11 januari 2015

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de bijbehorende toelichting, waarin elke stap in het beoordelingsproces wordt toegelicht

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD/801002015126
2. Titel van het project: Neurobiology of compulsive behavior and its components: Brain stimulation and measurements.
3. Titel van de NTS: Compulsief gedrag en zijn componenten: neurobiologische metingen en hersenstimulatie
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 19-06-2015
   - ✓ aanvraag compleet (herziening) ontvangen: 13-07-2015
   - ✓ in vergadering besproken: 29-06-2015
   - ✓ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en): n.v.t.
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ✓ advies aan CCD: 15-07-2015
7. Eventueel horen van aanvrager
   - Datum: n.v.t.
   - Plaats: n.v.t.
   - Aantal aanwezige DEC-leden: n.v.t.
   - Aanwezige (namens) aanvrager: n.v.t.
8. Correspondentie met de aanvrager:
   - Datum 30-06-2015
   - Strekking: suggesties voor completering van de aanvraag
   - Datum antwoord (gecompleteerde versie): 13-07-2015
   - Strekking van de antwoorden: de aanvraag is gecompleteerd
9. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig. Het omvat dierproeven in de zin der wet.
2. De aanvraag betreft een nieuwe aanvraag. Er is enige overlap met een aantal al van een positief advies voorziene DEC-protocollen.
3. De DEC is competent om over deze projectvergunningsaanvraag te adviseren. De benodigde expertise op dit wetenschappelijk terrein is aanwezig binnen de DEC. Geen van de DEC-leden is betrokken bij het betreffende project.
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering: n.v.t.

C. Beoordeling (inhoud):

1. Het project is wetenschappelijk verantwoord.

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.

3. De doelstelling en de uitvoering om de doelstelling te bereiken is door de indiener duidelijk omschreven in de aanvraag: Het met behulp van dierexperimenteel werk verkrijgen van fundamenteel-wetenschappelijke neurobiologische inzichten in het ontstaan van compulsief gedrag en in de verschillende componenten die ten grondslag liggen aan compulsiviteit. Het gebruik van de bestaande proefdiermodellen voor dwangmatig gedrag en de verdere ontwikkeling van nieuwe modellen zullen een beter inzicht geven in neuronale basis van een breed scala aan neuro-psychiatrische ziektebeelden in de mens zoals obsessief-compulsieve persoonlijkheidsstoornis (OCD), verslavingsgedrag en eetstoornissen.

   Het fundamenteel wetenschappelijke belang van het project acht de DEC substantieel: Compulsiviteit is betrokken bij een grote verscheidenheid aan gedragingen en afwijkingen in (componenten van) compulsiviteit en kunnen leiden tot ingrijpende gedragsstoornissen. Het verkrijgen van fundamentele wetenschappelijke kennis van de neuronale mechanismen die ten grondslag liggen aan compulsiviteit is van belang voor een beter inzicht in het functioneren van het gedrag van de mens. Bij het ontstaan van compulsiviteit-stoornissen zijn verschillende componenten betrokken zoals stress en angstgevoelens, de mate van gewoontevorming en het verlies van controle over doelgericht gedrag. Een betere onderbouwing van de hypothese dat de verschillende componenten en uitingen van compulsief gedrag gereguleerd worden door dezelfde of overlappende neurale circuits is van klinisch belang omdat ontregeling van deze circuits mogelijk een gemeenschappelijk oorzaak vormt voor uiteenlopende neuro-psychiatrische ziektebeelden. Inzicht in de manier waarop de verschillende hersengebieden in deze circuits bijdragen aan de regulering van (componenten van) compulsief gedrag zal niet alleen bijdragen aan een beter begrip van compulsiviteit-stoornissen in de maatschappij, het biedt ook kansen om deze stoornissen te corrigeren en bestaande therapieën (m.n. diepe hersenstimulatie) te verbeteren. Het project dient daarmee, op termijn, een belangrijk maatschappelijk belang.

4. De gekozen strategie, experimentele aanpak in combinatie met de infrastructuur op het [redacted] en de expertise van de betrokken onderzoeksgroep bieden

een realistisch uitzicht op het behalen van de beoogde doelstellingen binnen gevraagde looptijd van 5 jaar van het project. De onderzoeksgroep is ingebed in een grote klinische onderzoeksgroep van de afdeling psychiatrie van [redacted], hetgeen de uitwisseling van nieuwe kennis en inzichten tussen kliniek, klinisch onderzoek en het dierexperimentele werk in grote mate bevordert. Het reeds verrichte onderzoek van de groep heeft al belangrijke resultaten en publicaties opgeleverd en vormt een goede basis voor het voorgenomen onderzoek. Het gebruik van invasieve technieken met een hoge temporele en spatiële resolutie, om zo inzicht te krijgen in de relatie tussen neuronale activiteitspatronen en compulsief gedrag, is niet mogelijk in de mens. Beide onderzoekslijnen zullen naast elkaar worden uitgevoerd met een sterke onderlinge wisselwerking. Het dierexperimenteel onderzoek richt zich primair op een drietal verschillende diermodellen die reeds in het lab aanwezig zijn maar in het kader van het project zal ook worden onderzocht of andere modellen kunnen toegevoegd om zo de verschillende componenten van compulsief gedrag in optimale modellen te kunnen onderzoeken.

5. Alle dieren worden gefokt voor het gebruik in dierproeven, er is geen sprake van hergebruik. Zowel de mannelijke als vrouwelijke dieren worden gebruikt. Het is een noodzakelijk onderdeel van de proeven dat een deel van de dieren gedurende een korte of langere tijd solitair wordt gehuisvest. In die periode kunnen de dieren elkaar wel zien, horen en ruiken. Er is geen sprake van bedreigde diersoorten, niet-menselijke primaten, zwerfdieren en/of dieren in/uit het wild. De toegepaste methoden voor anesthesie/euthanasie zijn conform de Richtlijn.

6. Het cumulatieve ongerief gepaard gaand met de dierproeven, zoals beschreven in de vier verschillende type dierproeven, is naar inschatting van de DEC, voor het merendeel van de dieren matig (85% van de 1750 muizen en de 3250 ratten) en voor de overige dieren licht. Deze inschatting van de DEC is volledig in overeenstemming met het niveau van cumulatief ongerief zoals dat is geclassificeerd door de onderzoekers. Hun classificatie is gebaseerd op hun ervaring met de gebruikte modellen in vergelijkbare, al uitgevoerde, dierproeven.
   Er moet worden opgemerkt dat in sommige modellen het noodzakelijk is om aversieve stimulaties (bijvoorbeeld pijnlijke korte elektrische schokken) te gebruiken als onderdeel van de gedragstesten en dat pijnbestrijding in deze gevallen niet wordt toegepast omdat dit strijdig is met de doelstelling van het experiment. In alle andere gevallen wordt adequate pijnbestrijding gebruikt.

7. Binnen het project wordt maximaal gebruik gemaakt van methoden die de voorgestelde dierproeven geheel of gedeeltelijk **vervangen.**
   Een belangrijk onderdeel van de experimentele strategie is de wisselwerking tussen gedragsstudies en klinische (interventie)studies bij de mens en het dierexperimenteel onderzoek. De klinische resultaten zullen worden gebruikt om een gerichte keuze te maken uit een groot aantal mogelijke startpunten van het dierexperimenteel werk.
   Voor het verkrijgen van nieuw inzicht in basis van compulsiviteit gestuurd gedrag is onderzoek op neuronaal activiteitsniveau essentieel. Met de huidige stand van de techniek kan dit type onderzoek met een sterk invasief karakter niet (of slechts bij hoge uitzondering) in proefpersonen worden uitgevoerd. Naar het oordeel van de DEC zijn er geen alternatieven beschikbaar voor het voorgestelde gebruik van intacte dieren om te doelstelling van dit project te realiseren.

8. In het project wordt optimaal tegemoet gekomen aan de vereisten van **vermindering** van dierproeven. Het gebruik van zowel mannelijke als vrouwelijke dieren uit de fok draagt bij aan een reductie van aantal dieren gedood in voorraad maar kan in sommige gevallen ook de variatie in de metingen verhogen waardoor er wat meer dieren ongerief zullen ondervinden. Inzicht in sekseverschillen is echter ook onderdeel van de vraagstelling.
   De onderzoeksgroep heeft veel ervaring met dit type experimenten. Een belangrijk onderdeel van de experimentele strategie is de gefaseerde opzet zoals beschreven in onderdeel 3.4.3 en gevisualiseerd in de bijlage “flow chart”. Eerst wordt een nieuw model en de benodigde methoden om betrouwbare uitleesparameters met een zo laag mogelijke variabiliteit te behalen volledig geoptimaliseerd. Daarna wordt overgegaan tot vervolgexperimenten om (i) de neuronale activiteit gekoppeld aan het gedrag te bestuderen of (ii) een interventie strategie te bestuderen. Pas daarna zullen de neuronale activiteitsbepalingen en de interventies in een enkel dier worden bestudeerd. Op die manier wordt een empirische cyclus doorlopen, waarbij kennis uit voorgaande proeven leidt tot een optimaal design van de vervolgexperimenten, waardoor per experiment telkens niet meer dat het minimum aantal benodigde dieren wordt ingezet.
   Technieken en procedures worden zorgvuldig toegepast. Het totaal aantal te gebruiken dieren in het project is een realistische schatting, mede gebaseerd op de aantallen dieren gebruikt in het verleden in vergelijkbare experimenten.

9. De uitvoering van het project is in overeenstemming met de vereisten van **verfijning** van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.
   Bij de opzet wordt rekening gehouden met dierenwelzijn en wel op de volgende manieren: 1) het gebruik van adequate anesthesie en analgesie waar nodig/mogelijk, 2) toepassing van stress-verminderende procedures, 3) een intensieve monitoring van de proefdieren gecombineerd met duidelijk gedefinieerde humane eindpunten.

   Er moet worden opgemerkt dat in sommige van de modellen het noodzakelijk is om aversieve stimulaties (bijvoorbeeld het toedienen van korte elektrische schokken met een pijnlijk effect) te gebruiken als onderdeel van de gedragstesten en dat pijnbestrijding in deze gevallen niet wordt toegepast. De DEC acht dit onvermijdbaar voor het bereiken van het doel van het onderzoek (in alle andere gevallen wordt een adequate pijnbestrijding gebruikt).
   Daarnaast wordt in sommige proeven een deel van de dieren gedurende een korte of langere tijd solitair gehuisvest. In die periode kunnen de dieren elkaar wel zien, horen en ruiken. De DEC acht dit onvermijdbaar voor het bereiken van het doel van het onderzoek.

   Er is geen sprake van belangwekkende milieueffecten.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en is geformuleerd in begrijpelijke taal. De NTS voldoet daarmee aan de eisen zoals gesteld in artikel 10.a.1.7 van de Wod.

D. Ethische afweging

De centrale vraag voor de ethische afweging is of het belang van het doel van dit project opweegt tegen het ongerief dat de dieren ondergaan (geclassificeerd voor het merendeel van

de dieren als matig). Het doel van het project is het verkrijgen van fundamenteel wetenschappelijke inzichten in het ontstaan van compulsief gedrag en de verschillende componenten die ten grondslag liggen aan compulsiviteit. Het onderzoek is primair fundamenteel wetenschappelijk van karakter maar door een inbedding in een klinische onderzoeksgroep zijn bevindingen uit het dierexperimenteel werk ook direct toegankelijk voor een eventuele klinische toepassing. De verwachting is dat de resultaten van het onderzoek, op termijn, kunnen bijdragen aan een beter inzicht in de oorzaken van verschillende ziektebeelden waarbij een stoornis in compulsiviteit betrokken is. Het project dient daarmee, op termijn, *een belangrijk maatschappelijk belang*.

Het fundamenteel wetenschappelijke onderzoek in dit project is van hoge kwaliteit en het project is uit wetenschappelijk oogpunt verantwoord. De onderzoeksgroep beschikt over ervaring met de gekozen onderzoeksstrategie en met de voorgestelde typen dierproeven. De DEC is van mening dat de resultaten van de dierproeven zullen bijdragen aan het behalen van de geformuleerde doelstellingen en schat de kans op het realiseren van deze doelstellingen in als hoog. De verkregen fundamenteel wetenschappelijke kennis is onmisbaar om te komen tot een beter begrip van de neurobiologische mechanismen die een rol spelen in compulsiviteit en het project dient daarmee een *substantieel wetenschappelijk belang*.

Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van vervanging, vermindering en verfijning van de dierproeven. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald.
De DEC merkt op dat het een aanvraag betreft met de inzet van een groot aantal verschillende modellen voor compulsiteit en componenten van compulsiviteit gekoppeld aan verschillende meetmethoden van neuronale activiteit en methoden voor interventie. Dit schept een situatie waarbij een groot aantal verschillende combinaties mogelijk zijn. De DEC onderkent dat het voorgestelde onderzoek een sterk exploratief karakter heeft waarbij het moeilijk in te schatten is welke van de combinaties de grootste wetenschappelijke opbrengst zullen hebben. De randvoorwaarden zijn naar de mening van de DEC voldoende duidelijk vastgelegd zodat een ethische afweging mogelijk is. De IvD zal scherp moeten toezien dat de ingediende studieprotocollen binnen de afbakening van het projectvoorstel blijven.

De DEC komt tot de conclusie dat de doeleinden van het project het voorgestelde gebruik van de proefdieren en het daarmee samenhangende ongerief van de proefdieren rechtvaardigen.

E. Advies

1. Advies aan de CCD
   - ✓ **De DEC adviseert de vergunning te verlenen**
2. Het uitgebrachte advies is gebaseerd op consensus.
3. Er zijn geen knelpunten of dilemma's gesignaleerd tijdens het beoordelen van de aanvraag of het formuleren van het advies.

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Centrale Commissie Dierproeven

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2500 EK Den Haag
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T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Bijlagen
2

Datum 15-07-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning dierproeven

Geachte heer [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 15 juli 2015.
Het aanvraagnummer dat wij hieraan hebben gegeven is AVD801002015126. Gebruik dit nummer als u contact met ons opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te betalen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:

- Gegevens aanvraagformulier
- Factuur

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Onze referentie
Aanvraagnummer
AVD801002015126

Factuurdatum	15 juli 2015
Vervaldatum	15 augustus 2015
Factuurnummer	201570126
Betreft	Factuur Aanvraag projectvergunning dierproeven

Factuur

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD801002015126	€ 741,-

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL28RBOS 056.99.96.066 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 20401, 2500 EK te 's Gravenhage.

[redacted]

Van: secretariaat DEC [redacted]
Verzonden: woensdag 15 juli 2015 11:15
Aan: ZBO-CCD
Onderwerp: RE: indienen nieuwe PVA en CCD vergaderdatum en AVD-801002015126

Categorieën: [redacted]

Beste [redacted]

Nogmaals dank voor het doorgeven van deze nuttige informatie.
Ik heb zojuist alle documenten voor AVD-801002015126-[redacted] naar de CCD gestuurd via webftp. Het getekende aanvraagformulier wordt vandaag per post gestuurd.

Groet [redacted]
DEC-KNAW

From: ZBO-CCD [mailto:ZBO-CCD@minez.nl]
Sent: Monday, July 06, 2015 3:08 PM
To: secretariaat DEC
Subject: RE: indienen nieuwe PVA en CCD vergaderdatum

Beste meneer [redacted]

De optimale indiendata voor de verschillende CCD vergaderingen moeten nog door de CCD worden vastgesteld.
Ik kan daarop vooruitlopend wel aangeven dat de optimale indiendatum voor de volgende CCD vergadering [redacted] is.
Ik hoop u hiermee voorlopig voldoende te hebben geïnformeerd.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.zbo-ccd.nl**
Postbus 20401 | 2500 EK | Den Haag
T: 0900 2800028
E: ZBO-CCD@minez.nl

Van: secretariaat DEC [redacted]
Verzonden: vrijdag 3 juli 2015 14:54
Aan: ZBO-CCD
Onderwerp: indienen nieuwe PVA en CCD vergaderdatum

Geachte CCD-medewerker,

Ik zou van u graag de uiterste PVA- inleverdatum ontvangen voor de volgende CCD bijeenkomst. Het verstrekken van deze informatie is op de laatste bijeenkomst toegezegd door de CCD. Op dit moment hebben we een aantal PVA in behandeling en willen graag iets meer zicht op een optimale indiendatum.
Ik hoor graag van u.

Groet [redacted]
DEC-KNAW

[redacted]

Van: [redacted]
Verzonden: maandag 20 juli 2015 13:46
Aan: ZBO-CCD
CC: [redacted]
Onderwerp: Re: Aanvraag AVD801002015126

Categorieën: [redacted]

To whom it may concern at the Centrale Commissie Dierproeven,

I would like to make sure that you are aware that the payment for our application AVD801002015126 is being submitted as soon as possible. Our finance department ensures us that it will be made by Friday, July 24.

Thank you.

Best,
[redacted]

[redacted]
Group Leader & Principal Investigator
Team [redacted] Royal Netherlands Academy of Arts and Sciences (KNAW) &

[redacted] Amsterdam
The Netherlands

[redacted]

From: ZBO-CCD <ZBO-CCD@minez.nl>
Sent: Wednesday, July 15, 2015 1:43 PM
To: [redacted]
Cc: [redacted]
Subject: Aanvraag AVD801002015126

Geachte heer [redacted]

Deze brief is ook per post verzonden.

Met vriendelijke groet,

Centrale Commissie Dierproeven www.zbo-ccd.nl Nationaal Comité advies dierproevenbeleid
[redacted]
[redacted] Den Haag Postbus 20401 | 2500 EK | Den Haag

15

[redacted]

Van: Info-zbo
Verzonden: vrijdag 31 juli 2015 13:35
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD801002015126
Bijlagen: AVD801002015126_Vervolgbrief.pdf

Geachte heer [redacted]
Bijgevoegde brief is u vandaag ook per post verstuurd.

Met vriendelijke groet,

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**
Postbus 20401 | 2500 EK | Den Haag

Let op: vanaf nu heeft de CCD een nieuw e-mailadres info@zbo-ccd.nl. Heeft u ons oude e-mail adres in uw adressenboek, dan vragen we u om dat aan te passen.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Uw referentie
uw ref

Bijlagen
-

Datum 31-07-2015
Betreft Vervolg Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 15 juli 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neurobiology of compulsive behavior and its components: Brain stimulation and measurements" met aanvraagnummer AVD801002015126. Wij gaan uw aanvraag beoordelen. In deze brief leest u wanneer u een beslissing kunt verwachten.

Wanneer een beslissing
Wij nemen uiterlijk 08 september 2015 een beslissing. Omdat een DEC-advies is meegestuurd met de aanvraag, streven wij ernaar om de aanvraag binnen 20 werkdagen te beslissen.
Als wij nog informatie nodig hebben, kan dit later worden. Voor een complexe aanvraag staat een langere termijn. In beide gevallen ontvangt u daarover bericht. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Andere regelgeving
Hierbij wijzen wij u erop dat naast de regels die op de uitvoering van dierproeven van toepassing zijn op grond van de Wet op de dierproeven, er mogelijk ook verplichtingen kunnen voortvloeien uit andere wet- en regelgeving. In dit verband kan bijvoorbeeld worden gewezen op de Flora- en faunawet ten aanzien van in het wild levende dieren en de CITES-regelgeving ten aanzien van beschermde diersoorten.
Het besluit op uw aanvraag heeft alleen betrekking op de Wet op de Dierproeven en niet op andere wet- en regelgeving.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Uw referentie
uw ref

Bijlagen
-

Datum 31-07-2015
Betreft Vervolg Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

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Het besluit op uw aanvraag heeft alleen betrekking op de Wet op de Dierproeven en niet op andere wet- en regelgeving.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

[redacted]

Van: [redacted]
Verzonden: maandag 10 augustus 2015 15:35
Aan: 'Info-zbo'
CC: [redacted]'
Onderwerp: RE: Aanvullende informatie aanvraag AVD801002015126
Bijlagen: 2. AVD-801002015126 NTS revised.docx

Beste [redacted]

Dank voor je verzoek voor aanvullende informatie m.b.t. de NTS. Een punt van aandacht voor toekomstige PVA stukken.
Namens de onderzoeker stuur ik als bijlage bij deze mail een herziene versie van de NTS.
Graag een bevestiging van ontvangst.

Groet
[redacted] DEC-KNAW

From: Info-zbo <info@zbo-ccd.nl>
Sent: Monday, August 10, 2015 1:23 PM
To: [redacted]
Cc: Info-zbo
Subject: Aanvullende informatie aanvraag AVD801002015126

Beste Heer [redacted]
Zie bijgevoegde brief betreffende uw aanvraag AVD801002015126.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**
Postbus 20401 | 2500 EK | Den Haag
Let op: vanaf nu heeft de CCD een nieuw e-mailadres info@zbo-ccd.nl. Heeft u ons oude e-mail adres in uw adressenboek, dan vragen we u om dat aan te passen.

19

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Uw referentie
uw ref

Bijlagen
1

Datum 10 augustus 2015
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted],

Op 15 juli 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project “Neurobiology of compulsive behavior and its components: Brain stimulation and measurements” met aanvraagnummer AVD801002015126. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

Welke informatie nog nodig
Wij hebben de volgende informatie van u nodig om uw aanvraag verder te kunnen beoordelen:

Niet technische samenvatting
De niet technische samenvatting bij uw aanvraag bevat een verwijzing naar appendix 1 (bij vraag 3.6). De NTS moet zelfstandig leesbaar zijn, daarom aan u het verzoek om deze verwijzing te verwijderen.
Graag ontvangen wij een nieuwe versie van de Niet technische samenvatting.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Stuurt u het per post op, gebruik dan het formulier dat u bij deze brief krijgt.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Datum
10 augustus 2015

Onze referentie
Aanvraagnummer
AVD801002015126

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:

- formulier Melding Bijlagen via de post

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

- U wilt één of meerdere bijlagen naar ons versturen? Voeg *altijd* deze Melding Bijlagen toe. Wij weten dan welke documenten van u zijn en hoeveel documenten u opstuurt.
- Meer informatie vindt u op www.centralecommissiedierproeven.nl
- Of bel met ons: 0900 28 000 28 (10 ct/min).

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

1.2 Bij welke aanvraag hoort de bijlage?
Het aanvraagnummer staat in de brief of de ontvangstbevestiging.

Aanvraagnummer	

2 Bijlagen

2.1 Welke bijlagen stuurt u mee?
Vul de naam of omschrijving van de bijlage in.

☐	
☐	
☐	

3 Ondertekening

3.1 Onderteken het formulier en stuur het met alle bijlagen op naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Uw referentie
-

Bijlagen
1

Datum 12 augustus 2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 15 juli 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neurobiology of compulsive behavior and its components: Brain stimulation and measurements" met aanvraagnummer AVD801002015126. Wij hebben uw aanvraag beoordeeld.

Op 11 augustus 2015 heeft u uw aanvraag gewijzigd. Op ons verzoek heeft u een kleine wijziging aangebracht in de Niet technische samenvatting, zodat deze op zichzelf leesbaar is.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. Gelet op het gegeven dat de aanvraag dierproeven bevat die op grond van de oude regelgeving nog uitgevoerd mocht worden en deze proeven (opnieuw) ter beoordeling zijn voorgelegd, mogen deze proeven alleen nog worden uitgevoerd onder deze door de CCD afgegeven vergunning.
U kunt met uw project "Neurobiology of compulsive behavior and its components: Brain stimulation and measurements" starten. De vergunning wordt afgegeven van 12 augustus 2015 tot en met 01 augustus 2020. De looptijd van de vergunning wijkt af van uw aanvraag omdat de startdatum op uw aanvraag in het verleden ligt.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC KNAW gevoegd. Dit advies is opgesteld op 15 juli 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering.
Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze gegevens in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163

Bijlagen
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: KNAW
Adres: Postbus 19121
Postcode en woonplaats: 1000 GC Amsterdam
Deelnemersnummer: 80100

deze projectvergunning voor het tijdvak 12 augustus 2015 tot en met 01 augustus 2020, voor het project “Neurobiology of compulsive behavior and its components: Brain stimulation and measurements” met aanvraagnummer AVD801002015126, volgens advies van Dierexperimentencommissie DEC KNAW.
De functie van de verantwoordelijk onderzoeker is Group Leader.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 16 juli 2015
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 15 juli 2015;
   b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 11 augustus 2015 (herziene versie);
   c. Advies van Dierexperimentencommissie, ontvangen op 15 juli 2015

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst	Voorwaarden
Establishing and characterizing rodent behavior that models compulsive behavior and its component	Muis (Mus musculus) Rat (Rattus norvegicus)	500 muizen 1000 ratten	30% licht, 70% matig	Zie onder.
Identification of brain correlates of compulsive behavior and its components	Muis (Mus musculus) Rat (Rattus norvegicus)	350 muizen 700 ratten	matig	Zie onder
Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation	Muis (Mus musculus) Rat (Rattus norvegicus)	350 muizen 700 ratten	matig	Zie onder
Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation	Muis (Mus musculus) Rat (Rattus norvegicus)	150 muizen 150 ratten	matig	Zie onder
Identification and characterization of neuroanatomical connections and their regulation	Muis (Mus musculus) Rat (Rattus norvegicus)	400 muizen 700 ratten	300 muizen matig, 100 muizen licht. 525 ratten matig, 175 ratten licht	

Voorwaarden
Op grond van artikel 10a1 lid 2 Wet zijn aan een projectvergunning voorwaarden te stellen
De vergunning wordt verleend onder de voorwaarde dat eventuele go/no go momenten worden afgestemd met de IvD.

In artikel 10, lid 1a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Er is enige overlap met een aantal al van een positief advies voorziene DEC protocollen.
Bij ingang van deze vergunning mogen de proeven die zijn beschreven in eerder van positief advies voorziene DEC protocollen alleen nog worden uitgevoerd onder deze door de CCD afgegeven vergunning.

Weergave wet- en regelgeving

Dit project en wijzigingen

Volgens artikel 10c van de Wet op de dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister van Economische Zaken een ontheffing is verleend.

Verzorging

De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving

In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef

Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade

zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13c volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.

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Van: Info-zbo
Verzonden: donderdag 13 augustus 2015 8:40
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CC: [redacted]
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