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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
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: Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression using magnetothermal DBS

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 experimental approach of this study is to measure changes in extracellular levels of the main monoamines and changes in electrophysiological properties in potent brain regions during magnetothermal DBS of the different parts of the vmPFC [9, 2]. In our previous experiment we aim to discover activated brain regions during our stress protocol and DBS of the vmPFC, therefore involved in the microcircuit of depressive-like behavior. Quantification of these potent brain regions will take place using post-mortem brain tissue of our previous experimental group (appendix 3). Only the brain regions mostly activated during our animal model of depression and furthermore accessible for electrophysiology or microdialysis will undergo these measurements during their experiment. We will include sham control groups not receiving actual stimulation and sham control groups not experiencing stress from the CUS animal model.

As described in appendix 3, we will firstly sensitize neurons to heat in either the infralimbic, prelimbic or dorsal peduncular cortex using stereotactic injection of a lentivirus. This procedure takes place under general anesthesia. Additionally in part of the animals, we will insert a cannula in a brain region of interest that is involved in the microcircuits of depressive-like behaviour in our animal model. In each experimental group we will target a different brain region. Which regions are of interest is to be determined in our previous experimen. The implanted cannula will serve as an access point for microdialyses. Animals will be given a post-operative recovery period.

Secondly, we will gain depressive-like behaviour in animals using the chronic unpredictable stress (CUS)

model.

After a period of stress, determined in our first experiment, we will inject nanoparticles or a nanoparticle free solution under general anaesthesia using the same stereotactic coordinates as we did for injection of the lentivirus. We will inject in the same region as the lentivirus so either inject in the infralimbic, prelimbic or dorsal peduncular cortex. Sham control animals will be injected with a nanoparticle free solution.

After a post-operative recovery period we will start magnetothermal DBS by exposure to an alternating magnetic field. This alternating magnetic field causes a fast vibration of the magnetic nanoparticles. These fast movements generate heat and activate the heat-sensitive capsaicin receptors. Activation of these receptors causes neural excitation due to the influx of ions. All animals will undergo behavioural testing when exposed to the magnetic field. We will also add control groups not being exposed to a magnetic field. We will test antidepressant effects of magnetothermal DBS of subregions in the vmPFC by comparing behavioural outcomes between experimental and control groups. The maximal duration of stress including behavioural testing will be ten weeks.

In addition to magnetothermal DBS, microdialysis probes will be guided through the cannula and intracranial sampling of neurotransmitters will be performed. Measurements will take place before, during and after stimulation. The other part of the animals, not implanted with a cannula, will undergo electrophysiological recordings of different regions of interest under general anesthesia. So for all animals either microdialysis or electrophysiological measurements will be their final experiment followed by euthanasia with an overdose of pentobarbital and consecutive transcardial perfusion to preserve the brain for post-mortem analyses. We will measure shortly before, during and shortly after DBS. Which regions are of interest is to be determined in our previous experiment explained in appendix 1.

The primary outcomes:

- Changes in monoamine levels upon magnetothermal DBS of the different parts of the vmPFC.
- Changes in electrophysiological properties upon magnetothermal DBS of the different parts of the vmPFC.

We will investigate the most potent brain regions involved in the underlying microcircuit following magnetothermal DBS of the vmPFC observed in our previous experiment.

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

For this experimental design we will use experimental groups receiving the new technique of magnetothermal DBS in different regions of the vmPFC. Simultaneously we will measure the changes in monoamine concentrations levels and electrophysiological properties in different brain areas linked to stimulation of different parts of the vmPFC using microdialysis and electrophysiological recordings.

Surgery
We will inject lentivirus in either the infralimbic, prelimbic of dorsal peduncular cortex under general anesthesia using a stereotactic frame. With this lentivirus we sensitize neurons to heat by adding heat-sensitive receptors (TRPV) in the injected brain region. In a part of these animals, we will additionally insert a cannula in a brain region of interest for additional microdialysis measurements. A part of the animals will not be implanted with a cannula and will undergo electrophysiology instead of microdialysis. Animals will be given a post-operative recovery period.

Animal models
For our experiments we will use the CUS animal model. This model has been proven effective in mice and rats and is a well-validated animal models for experimental stress and depression [4, 7]. For the CUS model we will vary multiple unpredictable stressors during 3 consecutive weeks. These stressors will include a shift in hours of light per day, exposure to stroboscopic light (preferably 2.5 Hz), tilt cages, soiled bedding, paired housing and so on. During paired-housing, animals will be grouped in pairs with different partners. All stressors will last for 10-14 hours and since they should be unpredictable the exact

application times are not fixed. The stressors will always take place during the active period of the animal in a reversed day-night cycle in the laboratory. One will always take place in the morning while the other stressor will always take place in the afternoon or evening. We will continue these stressors during our DBS experiments. The maximal duration of stressor exposure will be ten weeks; including the days of experimental microdialysis or electrophysiology.

We are interested in the underlying mechanism of depression and its microcircuits; therefore the CUS models is the appropriate models to investigate our research questions.

Injection of nanoparticles
After the same duration of stress used in our previous experiments, we will inject nanoparticles or a nanoparticle free solution in the same region as we the injected lentivirus. This procedure is executed under general anaesthesia. Nanoparticles will be obtained from the same supplier stated in appendix 3. All animals will get a post-operative recovery period.

Magnetothermal DBS
After a post-operative recovery period, all animals will undergo magnetothermal DBS by exposure to a alternating magnetic field. In this method, specific neurons are sensitized to heat by lentiviral delivery of heat-sensitive receptors (TRPV). These receptors can be activated by magnetic nanopartides using an alternating magnetic field. This alternating magnetic field causes a fast vibration of the magnetic nanoparticles generating heat in the near surrounded area. This heat activates the heat-sensitive capsaicin receptors causing neural excitation due to the influx of ions. This makes magnetothermal DBS a perfect method to remotely and wirelessly drive neural excitation. Parameters of the stimulation paradigm will be obtained during our exchange and pilot studies at MIT and our previous experiments described in appendix 3.

Microdialysis and electrophysiology
In addition to magnetothermal DBS we will conduct microdialysis or electrophysiological recordings of different parts of the microcircuits discovered in our previous experiments. We will collect CSF samples via the cannula implanted during stereotactic surgery. In these samples we will analyse different monoamine concentrations to discover what changes during magnetothermal DBS of different parts of the vmPFC. Part of the animals will be sacrificed after microdialysis using an overdose of pentobarbital followed by transcardial perfusion. The other part of animals will undergo general anaesthesia for electrophysiological recordings of other potent brain regions found in our previous experiments. Since the animals are under general anaesthesia scanning multiple brain regions will not increase the level of discomfort. The amount of animals per group is to be determined in our previous experiments. This is their final experiment so after this experiment all animals will be sacrificed using an overdose of pentobarbital followed by transcardial perfusion.

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 needed, we considered the published study of magnetothermal DBS in mice and previous research done in our laboratory using current DBS in the vmPFC of rats and research performing microdialysis and electrophysiology of different brain regions in rats [6, 7, 9, 2].

B. The animals

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

Species

We will use adult male Sprague Dawley rats purchased from a registered breeding facility. We will use these animals since we have experience with them in our group, because they can be used in the CUS model and because microdialysis and electrophysiology can be performed on these animals [7, 9]. Beforehand, we will conduct pilot studies of magnetothermal DBS in awake rats in the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA) (not in this protocol). Rats provide much more reliable data compared to mice [3].

Gender

We will use only male rats since previous research has shown that the estrogen cycle of female rodents can interfere with behavior and the release of neurotransmitters [1, 10]. Their estrogen cycle will cause a larger spread in behavioral outcomes, which will interfere with our readout measurements [10].

Number of animals

The estimation of the number of animals needed per group is based on a pilot study of magnetothermal DBS done at MIT together with previous published research concerning the CUS model and microdialysis in parallel with electrophysiological measurement [8, 7, 9]. We will use a maximum of 15 animals per group. We will have three different stimulation regions (infralimbic cortex, prelimbic cortex and dorsal peduncular cortex).

Before starting experiments, we will perform a power analysis and take into account a possible drop out of animals observed in our previous experiments. Animal dropout during our experiments can be due to: surgical complications during injection of the lentivirus or injection of the nanoparticles, cannula loss, too much stress during the CUS model or complications during electrophysiology.

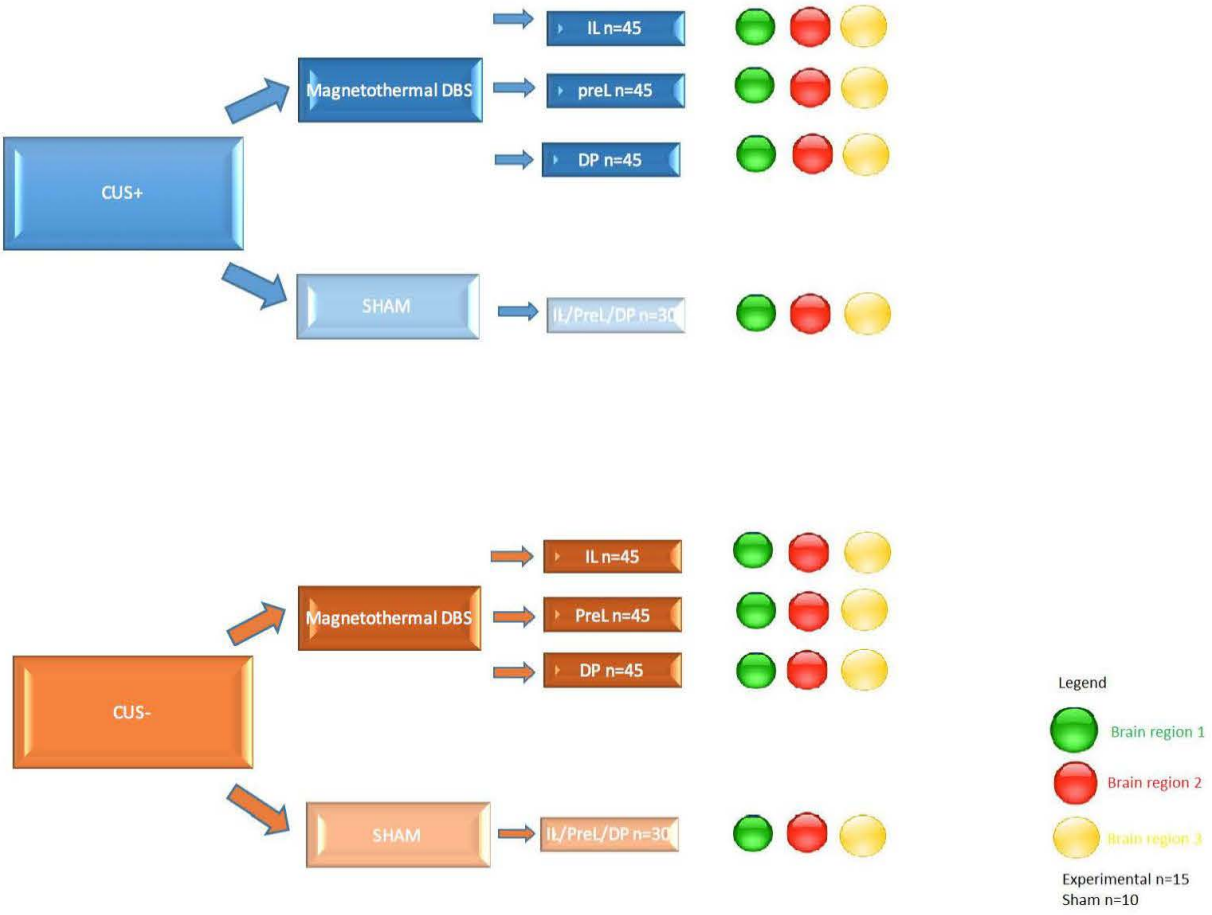
Since magnetothermal DBS for now has only been tested in mice, we need to extrapolate this method to rats. We are currently conducting pilot studies of magnetothermal DBS in rats.

This will lead to:

Microdialysis: the experimental group CUS+ with magnetothermal DBS (infralimbic, prelimbic, dorsopeduncular; n=45) in three potent brain regions (n=135), and their sham controls (n=30). And the naïve group CUS- with magnetothermal DBS (n=135) and their sham controls (n=30).

Electrophysiology: the experimental group CUS+ with magnetothermal DBS (infralimbic, prelimbic, dorsopeduncular; n=45) in three potent brain regions (n=135), and their sham controls (n=30). And the naïve group CUS- with magnetothermal DBS (n=135) and their sham controls (n=30).

Furthermore a possible 25% dropout needs to be taken into consideration. Taken all this into account we will use a maximal amount of 830 rats. Further reduction may follow in our working protocol after previous experiments has shown us the most potent regions of interest to measure chemical and electrophysiological changes.



igure 1. Subdivision of groups for Magnetothermal DBS and additional microdialysis (n=15) or electrophysiology (n=15), together with a sham control group (n=10). Each brain region indicated with a green, red or yellow circle, will either be a microdialysis experiment or an electrophysiological experiment.

C. Re-use

Will the animals be re-used?

x☐ 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.

Replacement

The primary aim of this study is to investigate the effect of infralimbic cortex, prelimbic cortex and dorsal peduncular cortex magnetothermal DBS on the levels of monoamines and

electrophysiological properties in different forebrain regions. This will help us to uncover the underlying mechanisms of antidepressant effects of the new technique of magnetothermal DBS in the vmPFC. This cannot be achieved using in vitro experiments or in computer models since we need a complex 'depressed' neural microcircuit to detect changes in monoamine levels and electrophysiological properties. This study cannot be performed in humans since this procedure with a lentivirus at this stage of research will not be ethically approved.

Reduction
We have chosen a validated and well-known model of experimental stress and depression used before in our laboratory. In this way we limit the number of animals in our study since differences in specific behavioural-parameters following current DBS has been shown before. We hypothesize that magnetothermal DBS will also have these effects and that underlying changes in monoamines and electrophysiological properties can therefore also be detected. We have limited the number of animals based on previous results of magnetothermal DBS in mice, current DBS in the vmPFC of rats and microdialysis together with electrophysiological measurements in different brain regions [6, 8, 9, 2]. The intermediate results of our previous studies could further reduce group size if possible. If previous experiments show that only particular forebrain regions are of interest to measure monoamine levels and electrophysiological properties we will only measure these specific brain regions.

Refinement
To refine magnetothermal DBS we will first train the experimental procedure under supervision of the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). To refine the procedures of microdialysis and electrophysiology we will firstly conduct these at the department of neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.). Both will reduce the amount of errors.
We will use general anesthesia during the insertion of the lentivirus, the insertion of nanoparticles, the insertion of the cannula for microdialysis and during electrophysiology. All animals will receive appropriate analgesics during post-operative recovery when needed. Too further refine our experiment we will insert different human endpoints.

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

All animals will be under general anaesthesia during; stereotactic injection of the lentivirus, stereotactic injection of magnetic nanoparticles into these brain regions later on, implementation of the cannula for microdialysis and during electrophysiological recordings. They will all get a post-operative recovery period. All animals will receive appropriate analgesics during their post-operative recovery period when needed. All animals will be euthanized with an overdose of pentobarbital before transcardial perfusion, either after microdialysis of electrophysiological recordings. To further minimize suffering in our animal models we will insert human endpoints in our protocol as described below.

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.

As stated and motivated in appendix 3, the procedure of magnetothermal DBS is very new and so far has only been preformed as a proof of principle at the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). It has not been used in an animal model for experimental depression. It would be the first experiment in which we will apply this new technique of DBS and look at the involved microcircuits in mood related behavior. By combining a new nanotechnique of DBS with experimental depression will provide a new strategy bringing the research field of neurosurgery, advanced nanotechnology and psychiatry closer together. We might be able to

develop a novel therapeutic strategy for patients with severe mood disorders and fine-tune neuromodulation using magnetothermal DBS.

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

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

All animals will be housed individually.

For the chronic unpredictable stress (CUS) model all animals will undergo variable stressors for the maximal duration of ten weeks. We will vary the stressors daily to induce chronic stress, which is the basis of our research. For this animal model indivual housing is nescesarry since it enhances susceptiblility to these chronic stressors [11].

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?

X ☐ 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.

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

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

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

General anesthesia will be used during stereotactic lentiviral delivery of heat sensitive receptor TRPV, stereotactic injection of magnetic nanoparticles into these brain regions, stereotactic implantations of a cannula for microdialysis and electrophysiological recordings.

Animals will receive appropriate analgesics during a post-operative recovery period. All animals will be monitored daily during the experimental procedures and will be checked for signs of pain or too much discomfort. Taking into consideration that here will be some discomfort using the CUS animal model. In case animals show pain during any of the experimental procedures described, they will receive analgesics.

All animals will get general anesthesia during euthanasia or during electrophysiological measurements.

Euthanasia will take place after microdialysis.

To further minimalize suffering we will insert human endpoints in our protocol as described below.

I. Other aspects compromising the welfare of the animals

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

All animals will experience chronic stress by adding variable stressors during our experimental model of depression. Our research is based on experimental depression and the previous mentioned validated animal model for experimental depression includes exposure to chronic stress.

Animals will experience mild additional stress during microdialysis.

Another adverse effect of stereotactic insertion of the cannula for microdialysis could be loss of a cannula construct.

Furthermore, there could be a possible higher risk of infection during the time between the injection of the lentivirus and the insertion of nanoparticles in the same region four weeks later.

Explain why these effects may emerge.

Stress during our experimental depression animal model will occur since we will apply daily stressors such as; tilt cages, soiled bedding or stroboscopic light. Additional stress will emerge when executing CSF sampling during microdialysis.

Loss of an electrode or cannula construct might be due to scratching.

After lentiviral injection we need to keep an access to the same brain region to inject nanoparticles four weeks later.

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

During our CUS model, all animals will be inspected daily. When an animal shows too much stress, i.e. a disrupted physical appearance together with disrupted unprovoked behaviour and or abnormal responses to external stimuli and or self-induced trauma, we will remove it from the experiment and euthanize it consecutively. This will be discussed in more detail in our humane endpoints and working protocol.

To prevent loss of a microdialysis cannula construct, we will secure them to the skull in the right position using dental cement. We will give all animals post-operative recovery-period that allows the skin around the cannula to recover. During our recovery period and experiment, all animals will be inspected daily.

To prevent infection we will cover up the injection side with a plastic cover slip and dental cement, which can be taken off during the injection of nanoparticles. Animals will be inspected daily during the post-operative recovery period and following experiments.

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.

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

Human endpoints will mainly be based on general considerations regarding general health, too much discomfort in the CUS animal model, complications of the injection of lentivirus into the brain or complications during stereotactic insertion of the nanoparticles.

Human endpoints will include:

-Signs of a disturbed physical appearance, disturbed unprovoked behavior, self-induced trauma or moderately abnormal responses to external stimuli.

-Rapid or progressive weight loss together with a loss of body condition compared to the beginning of our experiments. During our experiment we will inspect all animals daily using the general impression and physiological condition score format. We will weight all animals several times a week. Only measuring weight changes or disturbances in body condition is not a good way to interpret general health and therefore we will use both measurements scales together[5].

-Signs of untreatable local or generalized infection for more than 3 days: diarrhea, coughing and progressive weight loss.

- Major bleeding due to stereotactic lentiviral or nanoparticle injections.

If a human endpoint is reached we will take the animal out of the experiment, euthanize it with an overdose of pentobarbital, follwed by trasncardial perfusion for post-mortem analysis. At the end of the experiments, all animals will be sacrificed with an overdose of pentobarbital follwed by transcardial perfusion for post-mortem brain analysis.

Indicate the likely incidence.

We estimate the likely incidence at maximally 25%, for this reason we included this percentage of dropouts in our maximal estimated number of animals.

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').

All animals will undergo moderate discomfort following surgery.
75% will undergo severe discomfort during the induced depression and following experiments while 25% of all animals will undergo mild discomfort as they serve as non-depressed controls.

Surgery
The level of discomfort following stereotactic lentiviral TRPV delivery and stereotactic injection of nanoparticles four weeks later will be moderate. The insertion of the cannula for microdialysis will be moderate as well. All procedures will take place under general anaesthesia.

CUS animal model
The expected level of discomfort due to the CUS animal model used and individual housing is severe. This animal model of experimental depression will give a high level of stress during several consecutive weeks.

Wireless magnetothermal Deep brain stimulation
The level of discomfort following magnetothermal DBS will be mild.

Microdialysis:
The level of discomfort during microdialysis will be mild. We will collect samples of CSF before, during and after stimulation in free moving animals using the implanted cannula.

Euthanasia:
The level of discomfort during euthanasia with an overdose of pentobarbital followed by transcardial perfusion will be mild. Euthanasia will follow after microdialysis when electrophysiological measurements are no longer needed.

Electrophysiology:

The level of discomfort during electrophysiological measurements will be mild since all animals will be under general anaesthesia during this procedure. This procedure is followed by an overdose of pentobarbital and consecutive transcardial perfusion.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

All animals will be sacrificed at the end of the experiments right after electrophysiological measurements. We will isolate the brains to check for the lentiviral TRPV and nanoparticle localization, cannula and recording probe localisation and to perform immunohistochemistry in a later stage if needed.

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.

x☐ Yes

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2. Chen, R., G. Romero, M.G. Christiansen, A. Mohr, and P. Anikeeva, *Wireless magnetothermal deep brain stimulation*. Science, 2015. **347**(6229): p. 1477-80.
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4. Krishnan, V. and E.J. Nestler, *Animal models of depression: molecular perspectives.* Curr Top Behav Neurosci, 2011. **7**: p. 121-47.
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7. Lim, L.W., J. Prickaerts, G. Huguet, E. Kadar, H. Hartung, T. Sharp, and Y. Temel, *Electrical stimulation alleviates depressive-like behaviors of rats: investigation of brain targets and potential mechanisms*. Transl Psychiatry, 2015. **5**: p. e535.
8. Suzuki, S., A. Saitoh, M. Ohashi, M. Yamada, J.I. Oka, and M. Yamada, *The infralimbic and prelimbic medial prefrontal cortices have differential functions in the expression of anxiety-like behaviors in mice*. Behav Brain Res, 2016.
9. Tan, S.K., H. Hartung, V. Visser-Vandewalle, H.W. Steinbusch, Y. Temel, and T. Sharp, *A combined in vivo neurochemical and electrophysiological analysis of the effect of high-frequency stimulation of the subthalamic nucleus on 5-HT transmission*. Exp Neurol, 2012. **233**(1): p. 145-53.
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11. Willner, P., *Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS*. Neuropsychobiology, 2005. **52**(2): p. 90-110.

8.

DEC-advies PV-2015-016-[redacted]

A. Algemene gegevens over de procedure

1. Aanvraagnummer; 2015-016

2. Titel van het project; *Magnetothermal and current deep brain stimulation in experimental depression.*

3. Titel van de NTS; *Magnetothermale diepe hersenstimulatie (DBS) in een dier model voor depressie.*

4. Type aanvraag:

   X nieuwe aanvraag projectvergunning

   ☐ wijziging van vergunning met nummer

5. Contactgegevens DEC:

   - naam DEC; **DEC-UM**
   - telefoonnummer contactpersoon; [redacted]
   - mailadres contactpersoon; [redacted]

6. Adviestraject (data dd-mm-jjjj):

   X ontvangen door DEC; 09-03-2016

   ☐ aanvraag compleet;

   X in vergadering besproken; 18-03-2016

   ☐ anderszins behandeld;

   ☐ termijnonderbrekingen van 24-03-2016 tot 11-04-2016/14-04-2016 tot 29-04-2015/01-05-2016-05-05-2016

   ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen

   ☐ aanpassing aanvraag

   ☐ advies aan CCD

7. Eventueel horen van aanvrager **NVT**

8. Correspondentie met de aanvrager:

- Datum 23-03-2016

Strekking van de vragen:
3.2 Achtergrond: In uw project voorstel staan twee doelen beschreven. U wilt de rol van 3 microcircuits in de prefrontale cortex in relatie tot depressie middels stemming gerelateerd gedrag bestuderen. Daarnaast wilt u een nieuw ontwikkelde techniek van magnetothermale stimulatie testen in het wakkere dier, bij voorkeur in de rat. Omdat deze techniek nog in de kinderschoenen staat, moet het onderzoek naar de circuits in de vmPFC worden uitgevoerd met de gouden standaard "current DBS". Er is gekozen voor een verdubbeling van het aantal experimenten en proefdieren door de experimenten te herhalen met magnetothermale DBS. Om magnetothermale DBS te onderzoeken zou als mogelijk alternatief misschien ook de proefopzet gekozen kunnen worden, waarbij de gouden standaard (current DBS) een voorspelbaar effect zou bereiken. Mogelijk kunnen hierbij minder proefdieren gebruikt worden. Kunt u uw keuze voor de huidige proefopzet verder toelichten?
Tevens merkt de DEC-UM op dat de vmPFC en in het algemeen PC bij knaagdieren, nogal verschilt van de mens. In hoeverre denkt u dat de verschillende subregionen te herleiden zijn tot een vergelijkbare structuur bij de mens?

3.4 Onderzoeksstrategie: 3.4.1: Voorafgaand aan dit project worden pilot experimenten uitgevoerd om de magnetothermale methode voor de wakkere rat geschikt te maken.
Indien dit niet slaagt, wordt over gegaan naar het gebruik van de muis. De DEC-UM verzoekt de onderzoekers te onderbouwen waarom in een dergelijke situatie toch het translationele doel van het gebruik in de mens haalbaar is?

Op meerdere plaatsen in de tekst (PV en bijlage 1) en figuur 1 wordt de suggestie gewekt dat de pilot studie met magnetothermale stimulatie onderdeel is van dit project. Uit bijlage 3 blijkt dat deze pilot studie binnen MIT zal plaatsvinden.
De keuze van het proefdier model hangt af van het succes van de pilot experimenten.

Om de transparantie te vergroten verzoekt de DEC-UM om expliciet te vermelden dat of ratten of muizen gebruikt zullen worden en dus niet gebruik gemaakt zal worden van beide diersoorten.

3.4 Onderzoeksstrategie: 3.4.2: Hier wordt gesproken over "animal models en rodentia". De DEC verzoekt u eenduidig uw model te definiëren.

In uw onderzoeksprotocol maakt u gebruik van diverse gedragstesten om het stress niveau van het dier te bepalen. De DEC-UM vraagt zich af of de basale mate van stress in het dier, eventueel ook bepaald kan worden middels het bepalen van stress hormonen in het bloed? Zouden deze niveaus bepaald kunnen worden i.p.v. de gedragstesten, waardoor mogelijk een afname van ongerief zal bereiken?

3.4.4-appendix 1:

- De geslachtskeuze is onderbouwd met literatuur, maar hoe is de vertaling daarna naar de mens?
- De DEC-UM verzoekt u 25% uitval toe te lichten.
- CUS model klinkt "vriendelijk", maar volgens de DEC-UM worden de dieren chronisch depressief gemaakt, klopt dat en indien dat zo is, kunt u dit aangeven bij het ongerief? Is het ongerief wel juist ingeschat?
- Transcardiale perfusie is geen goedgekeurde dodingsmethode, van de DEC-UM kunt u hem toepasssen in het kader van het experiment, maar hij is niet officieel genoemd.
- De titel van appendix 1 verwijst naar current DBS. Tevens zijn de aantallen van de dieren hierop gebaseerd. Op meerdere plaatsen in deze appendix wordt echter magnetothermal DBS genoemd. Dit werkt verwarrend.
- De keuze voor alleen mannen is niet navolgbaar. Net als PD komt depressie niet alleen bij mannen voor. Daarom zijn mannelijke en vrouwelijke microcircuits en hun respons op deze nieuwe vorm van neuromodulatie beide interessant.
- Dieren worden individueel gehuisvest, omdat ze dan meer vatbaar zijn voor de stressoren. Ze worden er dus nog depressiever van. Is dat een aanvaardbare methode?
- De DEC-UM constateert dat het om een enorme hoeveelheid testen gaat, waarvan niet onderbouwd is dat ze allemaal nodig zijn. De DEC-UM wenst een onderbouwing welke experimenten u wanneer gaat doen.

3.4.4-appendix 2:

- Ook hier twee soorten controles. De DEC-UM zou hier graag meer duidelijkheid in willen zien. Zijn alle controlegroepen voor de beantwoording van uw onderzoeksvraag noodzakelijk?
- Transcardiale perfusie is geen goedgekeurde dodingsmethode in de ETS.
- Waarom wordt ervoor gekozen om deze dieren geen gedragsexperimenten te laten uitvoeren? De link van de microcircuits en (de mate van) depressie zal nu niet of moeilijk te bepalen zijn.

3.4.4-appendix 3:

- Het lentivirus en de nanopartikels worden in twee aparte procedures ingebracht. Zou het technisch haalbaar zijn dit in één procedure uit te voeren?
- Het ongerief voor magnetothermale DBS wordt ingeschat op mild. Waar is deze milde mate van ongerief op gebaseerd? De directe handelingen met het dier of de stimulatie zelf?

3.4.4-appendix 4:

- Sectie B. Species. *Rats and adult male mice* hier wordt waarschijnlijk **or** bedoeld.
- Waarom kiezen de onderzoekers er voor om deze dieren geen gedragsexperimenten te laten uitvoeren? De link van de microcircuits en (de mate van?) depressie zal nu niet te bepalen zijn.

NTS:

De DEC-UM verzoekt u, nadat u de vragen van de DEC-UM heeft beantwoord, ook de NTS daar waar nodig aan te passen.

- Datum antwoord: **dd. 11-04-2016**

- Strekking van de antwoorden: **Niet alle vragen zijn naar tevredenheid beantwoord.**
- De DEC-UM heeft opnieuw vragen gesteld **dd. 14-04-2016.**
- Antwoorden ontvangen **dd. 29-04-2016**
  De vragen zijn naar tevredenheid beantwoord. Er dienen echter nog een aantal kleine tekstuele aanpassingen gedaan te worden.
- De antwoorden hebben geleid tot aanpassing van de aanvraag; **JA**
- Stukken compleet en juist aangeleverd dd. 05-05-2016.

9. Eventuele adviezen door experts (niet lid van de DEC) **NVT**

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet); **JA**
2. De aanvraag betreft een **nieuwe aanvraag.**
3. De DEC is competent om hierover te adviseren; **JA**
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering

C. Beoordeling (inhoud):

1. Het project is:

   X uit wetenschappelijk oogpunt verantwoord
   ☐ uit onderwijskundig oogpunt verantwoord
   ☐ uit het oogpunt van productiedoeleinden verantwoord
   ☐ wettelijk vereist

2. De in de aanvraag aangekruiste doelcategorieën zijn in overeenstemming met de hoofddoelstellingen. **JA**
3. De DEC onderschrijft het belang van de doelstelling. Het wordt ingeschat als een **substantieel** belang.
4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. **JA**

5. Er is sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren. De keuze hiervoor is voldoende wetenschappelijk onderbouwd. **NVT**
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. **JA**
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.**
8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de **vermindering** van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat. De aanvrager gaat bij een bekende onderzoeksgroep, waar de techniek al uitgevoerd wordt, te rade om zich te bekwamen, waardoor het aantal dieren gebruikt in het huidige experiment, reëel is ingeschat. De DEC-UM heeft hier voorts geadviseerd, in nauw overleg met de IVD, het aantal gedragstesten te minimaliseren, omdat meerdere gedragstesten hetzelfde gedrag testen.
9. Het project is in overeenstemming met de vereiste van de **verfijning** van dierproeven en het project is zo opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Er is geen sprake van belangwekkende milieueffecten.

   Door toepassing van de juiste anesthesie en pijnbestrijding wordt zoveel mogelijk tegemoet komen aan de nodige verfijning. Ook worden er humane eindpunten toegepast waardoor onnodig lijden, in het kader van dit experiment, in een vroeg stadium wordt erkend, herkend.
10. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd. **JA**

D. Ethische afweging

De DEC-UM heeft het project *"Magnetothermal and current deep brain stimulation in experimental depression"* bestudeerd. Het behelst terminale experimenten met matig en ernstig ongerief gedurende 5 jaar, met maximaal 1950 ratten. Anderzijds onderschrijft de DEC-UM de intrinsieke waarde van het dier.
De DEC-UM is overtuigd van de wetenschappelijke waarde van het voorgestelde onderzoeksproject. Bevindingen in dit onderzoek kunnen mogelijkerwijze ook van maatschappelijk belang zijn, als de conclusies uiteindelijk leiden tot verbeterde behandeling van depressie. Derhalve is het terecht geclassificeerd als fundamenteel onderzoek.

Het project beoogt een beter neurobiologisch begrip op te leveren van de specifieke regionen en microcircuits in de hersenen, die betrokken zijn bij aandoeningen zoals depressie. Er wordt voortgebouwd op bevindingen in wetenschappelijk onderzoek dat Deep Brain Stimulation (DBS) bij obsessieve-compulsieve stoornissen verlichting geeft van bijkomende depressieve symptomen.
De onderzoekers willen tevens mechanismen rondom stemmings gerelateerd gedrag identificeren die kunnen worden beïnvloed door neuromodulatie met behulp van DBS. De huidige wijze van DBS is weliswaar de gouden standaard op het gebied van neuromodulatie, maar heeft nadelen. Vandaar dat de onderzoekers een recent ontwikkelde, meer verfijnde vorm van neuromodulatie ermee willen vergelijken, magnetothermale DBS.
Resultaten van dit onderzoek dragen in eerste instantie bij aan wetenschappelijke kennis van neurologische en psychiatrische aandoeningen. Er worden ook mogelijke behandelwijzen onderzocht. Belanghebbenden zijn daarom uiteindelijk ook de betreffende patiënten en hun omgeving. De DEC-UM acht derhalve de doelstelling van dit onderzoek van substantieel belang.
De opzet van het onderzoeksproject is helder, logisch en navolgbaar. De doelstellingen van de diverse onderdelen en de stapsgewijze aanpak, zijn overtuigend. De aanvrager beschikt over de benodigde wetenschappelijke kennis en technische expertise. Er wordt samengewerkt met deskundigen in Oxford en Boston en daar wordt de eventueel ontbrekende kennis en kunde aangevuld. Er is geen sprake van duplicatie. De gewenste uitkomsten zijn relevant in het licht van de overkoepelende vraagstelling en zijn ook haalbaar.
In de gekozen strategie, technieken en diermodellen wordt op bevredigende wijze tegemoet gekomen aan de vereisten op het gebied van vervanging, vermindering en verfijning. De DEC-UM is ervan overtuigd dat er geen alternatieven zijn, waardoor deze dierproef met minder ongerief of minder dieren zou kunnen worden uitgevoerd, dan wel het gebruik van levende dieren zou kunnen worden vermeden.
Op grond van deze argumenten acht de DEC-UM in haar ethische afweging het belang van project 2015-016 *"Magnetothermal and current deep brain stimulation in experimental depression"* van zwaarder gewicht dan de voorziene schade (25% matig ongerief, 75% ernstig ongerief en dood) voor de maximaal 1950 betrokken dieren. De DEC-UM beschouwt de voorgestelde dierproeven derhalve als ethisch gerechtvaardigd en voorziet het projectvoorstel *"Magnetothermal and current deep brain stimulation in experimental depression"* van een positief advies.

E. Advies

1. Advies aan de CCD

   X De DEC adviseert de vergunning te verlenen.

2. Het uitgebrachte advies is gebaseerd op consensus.

Op grond van alle voor de afweging relevante argumenten komt de DEC-UM tot de conclusie dat dit onderzoek ethisch toelaatbaar is.

Centrale Commissie Dierproeven

9.

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Onze referentie
Aanvraagnummer
AVD107002016542

Bijlagen
2

Datum 11 mei 2016
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 10 mei 2016.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD107002016542. 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. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u 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 voldoen, 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.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10700
Naam instelling of organisatie:	Universiteit Maastricht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	50169181
Straat en huisnummer:	Minderbroedersberg 4-6
Postbus:	616
Postcode en plaats:	6200 MD MAASTRICHT
IBAN:	NL04INGB0679510168
Tenaamstelling van het rekeningnummer:	Universiteit Utrecht

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]

Gegevens verantwoordelijke uitvoering proces

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 juni 2016
Geplande einddatum:	1 juni 2021
Titel project:	Magnetothermal and current deep brain stimulation in experimental depression
Titel niet-technische samenvatting:	Magnetothermale diepe hersenstimulatie (DBS) in een dier model voor depressie
Naam DEC:	DEC-UM
Postadres DEC:	Postbus 616, 6200 MD Maastricht
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 1.584,-
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:	Maastricht
Datum:	9 mei 2016

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD107002016542

Bijlagen
2

Datum 11 mei 2016
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 11 mei 2016
Vervaldatum: 10 juni 2016
Factuurnummer: 16700542

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD107002016542	€ 1.584,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL41RBOS 056.999.6317 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

10.

Antwoord onderzoeker op enkele vragen gesteld door de DEC

3.2 **Achtergrond:** In uw project voorstel staan twee doelen beschreven. U wilt de rol van 3 microcircuits in de prefrontale cortex in relatie tot depressie middels stemming gerelateerd gedrag bestuderen. Daarnaast wilt u een nieuw ontwikkelde techniek van magnetothermale stimulatie testen in het wakkere dier, bij voorkeur in de rat. Omdat deze techniek nog in de kinderschoenen staat, moet het onderzoek naar de circuits in de vmPFC worden uitgevoerd met de gouden standaard "current DBS". Er is gekozen voor een verdubbeling van het aantal experimenten en proefdieren door de experimenten te herhalen met magnetothermale DBS. Om magnetothermale DBS te onderzoeken zou als mogelijk alternatief misschien ook de proefopzet gekozen kunnen worden, waarbij de gouden standaard (current DBS) een voorspelbaar effect zou bereiken. Mogelijk kunnen hierbij minder proefdieren gebruikt worden. Kunt u uw keuze voor de huidige proefopzet verder toelichten?
Tevens merkt de DEC-UM op dat de vmPFC en in het algemeen PC bij knaagdieren, nogal verschilt van de mens. In hoeverre denkt u dat de verschillende subregionen te herleiden zijn tot een vergelijkbare structuur bij de mens?

1e Reactie onderzoeker:

Current DBS is een aspecifiek signaal en om deze reden is het niet mogelijk een voorspelbaar effect te bereiken. We willen de methoden 'magnetothermal DBS' en ' current DBS' vergelijken om te onderzoeken of het een mogelijke vervanger kan zijn voor current DBS en indien dit niet mogelijk is om stimulatie parameters voor current DBS te verbeteren. Om deze redenen is het gekozen voor de huidige proefopzet.

Er is in menig literatuur beschreven dat er gelijkenissen zijn tussen de prefrontale cortex van knaagdieren en mensen. Zo komt in de vmPFC het infralimbische gebied (IL) overeen met Brodmann's area 25 in de mens en het prelimbische gebied (preL) overeen met Brodmann's area 32 in de mens [1,2]. Ook andere hersengebieden die wij onderzoeken kent gelijkenissen met de mens, bijvoorbeeld de raphe nucleus [3]. Verschillende subregionen van de vmPFC en dieper gelegen hersenkernen bij knaagdieren zijn zodoende te herleiden tot vergelijkbare structuren in de mens.

[1] Myers-Schulz B, Koenings M. Functional anatomy of ventromedial prefrontal cortex: implications for mood and anxiety disorders. Mol Psychiatry. 2012 Feb;17(2):132-41. doi: 10.1038/mp.2011.88. Epub 2011 Jul 26.

[2] C Hamani, Y Temel. Deep Brain Stimulation for Psychiatric Disease: Contributions and Validity of Animal Models. Science Translational Medicine *11 Jul 2012: Vol. 4, Issue 142, pp. 142rv8*

[3] Mukherjee J et al. Comparative assessment of (18) F-Mefway as a serotonin 5-HT1A receptor PET imaging agent across species:Rodents, nonhuman primates, and humans. J. Comp Neurol. 2016 May 1;524(7):1457-71. doi: 10.1002/cne.23919. Epub 2015 Nov 18.

2e Reactie onderzoeker:

In de aangegeven literatuur heeft DBS van de vmPFC in ratten wel degelijk een effect op experimentele depressie en depressief-lijkend gedrag. Vermindering van depressief-lijkend gedrag is geconstateerd in meerdere gedragstaken waarbij verschillende modaliteiten van depressief gedrag werd getest (1).

Onderliggende microcircuits in de vmPFC hebben wel degelijk een effect op experimentele depressie. Echter is bij electrode implantatie geen onderverdeling gemaakt in de verschillende subregionen van de vmPFC, hetgeen we met dit onderzoek juist wel willen doen. Dit maakt het hopelijk mogelijk een onderscheidt te maken in specifieke verbeterde gedragstaken gekoppeld aan verschillende modaliteiten van depressie, mogelijk geïnduceerd door verschillende microcircuits van de vmPFC.
Current DBS en magnetothermal DBS hebben beide een verschillend werkingsmechanisme, de eerste werkt met toepaste stroom terwijl magnetothermal DBS werkt met activatie van eiwitten. Hierom is het nodig alle verschillende uitkomsten met elkaar te vergelijken en kunnen we niet alleen 1 microcircuit van current DBS testen met magnetothermal DBS. We verwachten tevens ook niet 1 microcircuit met een grootste effect, maar microcircuits met andere effecten in andere modaliteiten van depressie.

[1] Lim L.W., Temel Y. et al 'Electrical stimulation alleviates depressive-like behaviors of rats: investigation of brain targets and potential mechanisms.' Translational Psychiatry (2015), 1 – 14

3.4.4-appendix 1:
• De geslachtskeuze is onderbouwd met literatuur, maar hoe is de vertaling daarna naar de mens?
• De DEC-UM verzoekt u 25% uitval toe te lichten.
• CUS model klinkt "vriendelijk", maar volgens de DEC-UM worden de dieren chronisch depressief gemaakt, klopt daten indien dat zo is, kunt u dit aangeven bij het ongerief? Is het ongerief wel juist ingeschat?
• Transcardiale perfusie is geen goedgekeurde dodingsmethode, van de DEC-UM kunt u hem toepassen in het kader van het experiment, maar hij is niet officieel genoemd.
• De titel van appendix 1 verwijst naar current DBS. Tevens zijn de aantallen van de dieren hierop gebaseerd. Op meerdere plaatsen in deze appendix wordt echter magnetothermal DBS genoemd. Dit werkt verwarrend.
• De keuze voor alleen mannen is niet navolgbaar. Net als PD komt depressie niet alleen bij mannen voor. Daarom zijn mannelijke en vrouwelijke microcircuits en hun respons op deze nieuwe vorm van neuromodulatie beide interessant.
• Dieren worden individueel gehuisvest, omdat ze dan meer vatbaar zijn voor de stressoren. Ze worden er dus nog depressiever van. Is dat een aanvaardbare methode?
• De DEC-UM constateert dat het om een enorme hoeveelheid testen gaat, waarvan niet onderbouwd is dat ze allemaal nodig zijn. De DEC-UM wenst een onderbouwing welke experimenten u wanneer gaat doen.

3.4.4-appendix 1:

- De geslachtskeuze is onderbouwd met literatuur, maar hoe is de vertaling daarna naar de mens?
  Het is voor ons onderzoek van belang een zo uniform en controleerbaar mogelijk model te ontwerpen met zo min mogelijk variatie. Dit is onderbouwt met literatuur. Voor translatie naar de mens zijn mogelijk vervolgstudies nodig en dit onderzoek is dan ook een beginstapje naar het onderliggende circuit van depressie vallend onder basic research (zoals het project voorstel is ingediend).

- De DEC-UM verzoekt u 25% uitval toe te lichten.
  In onze onderzoeksgroep varieert de drop-out bij DBS in knaagdieren van 10-25%. Dit is afhankelijk van het goed blijven zitten van de electroden tijdens het CUS model, de duur van het experiment en de uit te voeren proceduren tijdens een experiment. Mijn beide voorgangers, met ervaring met het CUS model in ratten, hadden een drop-out van 10-25% welke ik gelijk hou voor in mijn onderzoek. Zodoende heb ik een maximale dropout van 25% en probeer deze gedurende de experimenten zo ver mogelijk te reduceren door chirurgische ingrepen en verdere proceduren zo veel mogelijk te optimaliseren.

- CUS model klinkt "vriendelijk", maar volgens de DEC-UM worden de dieren chronisch depressief gemaakt, klopt dat en indien dat zo is, kunt u dit aangeven bij het ongerief? Is het ongerief wel juist ingeschat?
  Het ongerief ervaren in het CUS model is inderdaad te laag ingeschat. Di tis veranderd naar severe ongerief.

- Transcardiale perfusie is geen goedgekeurde dodingsmethode, van de DEC-UM kunt u hem toepasssen in het kader van het experiment, maar hij is niet officieel genoemd.
  Transcardiale perfusie aangepast naar overdosis pentobarbital.

- De titel van appendix 1 verwijst naar current DBS. Tevens zijn de aantallen van de dieren hierop gebaseerd. Op meerdere plaatsen in deze appendix wordt echter magnetothermal DBS genoemd. Dit werkt verwarrend.
  Magnetothermal DBS wordt nu alleen nog genoemd voor het go/no-go moment onder het kopje 'species' sectie B en de berekening van het totaal aantal dieren.

- De keuze voor alleen mannen is niet navolgbaar. Net als PD komt depressie niet alleen bij mannen voor. Daarom zijn mannelijke en vrouwelijke microcircuits en hun respons op deze nieuwe vorm van neuromodulatie beide interessant.
  Het is voor ons onderzoek van belang een zo uniform en controleerbaar mogelijk model te ontwerpen met zo min mogelijk variatie. Dit is onderbouwt met literatuur. Dit onderzoek is een beginstapje naar het onderliggende circuit van depressie vallend onder basic research (zoals het project voorstel is ingediend). Hier. Hierbij is zo min mogelijk variatie gewenst. Voor translatie naar de mens en ook onderscheid in geslacht zijn vervolgstudies nodig.

- Dieren worden individueel gehuisvest, omdat ze dan meer vatbaar zijn voor de stressoren. Ze worden er dus nog depressiever van. Is dat een aanvaardbare methode?
  Dezelfde methode is verricht in voorgaand onderzoek in ons laboratrium, we gaan door op de resultaten uit dit onderzoek en houden het eerder uitgevoerde CUS model gelijk . In het CUS is individuele huishouding nodig. 1 van de stressoren waaraan de dieren in het CUS model worden blootgesteld is namelijk 'paired housing'. Het toedienen van de stressoren tezamen met het individueel huisvesten geeft het depressie niveau wat we willen bereiken.

- De DEC-UM constateert dat het om een enorme hoeveelheid testen gaat, waarvan niet onderbouwd is dat ze allemaal nodig zijn. De DEC-UM wenst een onderbouwing welke experimenten u wanneer gaat doen.
  We hebben een vierjarig PhD traject en gezien het vernieuwde, lange traject van een project aavraag hebben we besloten eerst appendix 1 uit te voeren tezamen met magnetothermal experimenten op MIT. Vervolgens voeren wij appendix 2, 3 en 4 uit zoals aangegeven in figuur 1 van het Project voorstel. Nu ook in elke appendix met een figuur verder toegelicht, De experimentele groepen zijn na nader overleg aangepast naar n=15 gezien er tijdens het CUS model repsonders en non-responders zijn. Er zijn dus iets grotere groepen nodig voor een behavioral em immunohistochemisch effect.
  Welke experimenten we wanneer precies zullen uitvoeren em met hoeveel dieren behoort volgens de nieuwe regeling echter thuis in een 'working protocol' welke zal volgen na de 'Project license'.

[4] Lim, L.W., J. Prickaerts, G. Huguet, E. Kadar, H. Hartung, T. Sharp, and Y. Temel, Electrical stimulation alleviates depressive-like behaviors of rats: investigation of brain targets and potential mechanisms. Transl Psychiatry, 2015. **5**: p. e535.

11.

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Onze referentie
Aanvraagnummer
AVD107002016542

Uw referentie

Bijlagen

Datum 31 mei 2016
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 10 mei 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Magnetothermal and current deep brain stimulation in experimental depression' met aanvraagnummer AVD107002016542. 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:

- In bijlage dierproeven 1 schrijft u: 'And the naive group CUS- with current DBS (n=30) and their sham controls (n=10)', maar in het schema en in de bijlage dierproeven 3 geeft u aan 45 ratten in de CUS- met current DBS nodig te hebben. We verzoeken u deze vergissing te corrigeren.
Daarnaast, volgens u aangegeven berekeningen per dierproef, komen we tot een aantal 138 ratten voor dierproeven 1 en 3 en tot 825 ratten in dierproeven 2 en 4 en niet tot 140 respectief 830 ratten vermeld in de aanvraag. Dat geeft een totaal van 1926 ratten en niet 1950, wat in de NTS vermeld is. We verzoeken u de aantallen aan te passen, of uw berekeningen te onderbouwen.

- In uw aanvraag schrijft u in de dierproeven 2 en 4 rekening te houden met de meest actieve regio's in dierproeven 1 en 3. Heeft u ook andere go/no-go of beslismomenten in uw opzet opgenomen? U wordt verzocht in het kader van vermindering go/no-go momenten vast te stellen (zowel bij de start van een dierproef, als tussen de verschillende dierproeven), de criteria te beschrijven op welke wijze bepaald zal worden of wel/niet met een dierproef gestart gaat worden?

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Gebruik hierbij het formulier dat u bij

deze brief krijgt indien u uw antwoord per post verstuurt. Om uw aanvraag in de eerstkomende vergadering te kunnen bespreken verzoeken we u vriendelijk om uiterlijk donderdag 9 juni 2016 uw antwoord naar ons toe te sturen.

Wanneer een beslissing
De behandeling van uw aanvraag wordt 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.

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	

12.

Datum 06 juni 2016

Betreft Antwoord op Aanvulling Aanvraag projectvergunning dierproeven

Geachte Centrale Commissie Dierproeven,

Hartelijk dank voor uw reactie ten aanzien van onze project aanvraag: AVD107002016542. Graag lichten we door middel van deze brief uw vraagstukken nader toe. De aangepaste tekststukken zijn grijs gemarkeerd in onze projectaanvraag terug te vinden.

De dier aantallen genoemd in appendices 1 t/m 4 en de 'non-technical summary' zijn aangepast. Appendix 1 en 3 zijn aangepast naar maximaal 138 ratten en appendix 2 en 4 naar maximaal 825 ratten hetgeen tezamen uitkomt op maximaal 1926 ratten.

De tweede vraag betreffende go/no-go momenten behoeft extra uitleg, welke we vraag middels deze brief geven.

De meest actieve hersenregio's na stimulatie van de vmPFC zullen worden vastgesteld in de dierproeven 1 en 3. Vooraf kunnen we niet voorspellen welke hersengebieden dit zijn. Wel stellen we vast alleen naar de drie meest potente gebieden te kijken mochten er meerdere interessante hersengebieden ontdekt worden door middel van post mortem brein analyse. Mochten we maar 1 of 2 interessante hersengebieden vinden dan onderzoeken we uiteraard alleen dit ene hersengebied of de twee interessante hersengebieden in de sequentiële appendices /dierproeven 2 en 4. Dit staat nu duidelijker beschreven in appendices 2 en 4.

We stimuleren de drie subregionen van de ventromediale prefrontale cortex (vmPFC; IL, PreL, DP) elk afzonderlijk omdat we van mening zijn dat stimulatie van deze verschillende subregio's, verschillende microcircuitries in gang zetten hetgeen zal leiden tot verschillende gedragsveranderingen. Depressie kent meerdere modaliteiten; anxiety, motivation, hedonia en behavioral despair, welke gekoppeld kunnen worden aan bepaald gedrag. Met stimulatie van deze verschillende subregionen en hun bijhorende microcircuit willen we onderscheid kunnen maken in deze modaliteiten van depressie. Dit doen we doormiddel van verschillende gedragstaken, elk passend bij een andere modaliteit van depressie. Dit kan van invloed zijn op de toekomstige behandeling van depressie waarin men gericht de voornaamste klacht van de patiënt behandeld door middel van modulatie van 1 specifieke subregio verantwoordelijk voor de depressieve gevoelens en het bijhorende gedrag. We kunnen ons verdere onderzoek dan ook niet continueren met maar 1 subregio van de vmPFC en hebben alle genoemde data van de in de appendices genoemde subgroepen nodig.

We kunnen geen go/no-go moment inbouwen om alleen 1 specifiek hersengebied van de vmPFC te onderzoeken met magnetothermale DBS. Het werkingsmechanisme van magnetothermale DBS en current DBS zijn dusdanig verschillend dat we geen voorspelbaar effect met current DBS kunnen bereiken voor de toepassing van magnetothermale DBS. Voor ons onderzoek is het nodig beide methoden in de drie vermelde subregionen van de vmPFC te onderzoeken. Mocht current DBS geen goed onderscheidt kunnen maken in de verschillende regionen en microcircuitries, dan kan magnetothermale DBS dit misschien wel gezien de hogere sensitiviteit en specificiteit van deze techniek. Voor dit alle zijn alle genoemde subgroepen van dieren in de appendices nodig. Een subgroep weglaten zal leiden tot onvolledigheid van het onderzoek en dus geen goed gegrond wetenschappelijk onderzoek met harde conclusies en goede vergelijkingen.

Wel hebben we de dieraantallen in appendices/dierproeven 2 en 4 verminderd door eerst een batch van n=8 dieren te onderzoeken. Mochten we met deze aantallen genoeg effect met microdialyse zien dan testen we alleen 8 ratten per subgroep. We hebben minimaal 18 neuronen nodig voor een elektrofysiologisch effect na stimulatie. Ook hier testen we eerst een batch van n=8 dieren. Mochten we in deze batch het minimaal aantal neuronen bereiken en genoeg materiaal hebben voor een elektrofysiologische analyse dan testen we alleen 8 ratten per subgroep. Dit kan zorgen voor een vermindering van het aantal ratten met n=390 in appendix 2 en n=390 in appendix 4. Ook deze veranderingen zijn met grijs in de appendices 2 en 4 weergegeven.

Hopelijk u hiermee voldoende te hebben geïnformeerd.

Met vriendelijke groet,

[redacted]
aanvragers projectvoorstel AVD107002016542.

Centrale Commissie Dierproeven

13.

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*	10700
☐ Nee > *U kunt geen aanvraag doen*	

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

Naam instelling of organisatie	Universiteit Maastricht	
Naam van de portefeuillehouder of diens gemachtigde	[redacted]	
KvK-nummer	50169181	
Straat en huisnummer	Minderbroedersberg	4-6
Postbus	616	
Postcode en plaats	6200 MD	Maastricht
IBAN	NL04 INGB 0679 5101 68	
Tenaamstelling van het rekeningnummer	Universiteit Maastricht	

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.

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☒ Dhr. ☐ Mw.
Functie	[redacted]	
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	[redacted]	☐ Dhr. ☒ Mw.
Functie	[redacted]	
Afdeling	[redacted]	
Telefoonnummer	[redacted]	
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	1 - 6 - 2016
Einddatum	1 - 6 - 2021

3.2 Wat is de titel van het project?

Magnetothermal and current deep brain stimulation in experimental depression

3.3 Wat is de titel van de niet-technische samenvatting?

Magnetothermale diepe hersenstimulatie (DBS) in een dier model voor depressie

3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?

Naam DEC	DEC-UM
Postadres	Postbus 616, 6200 MD Maastricht
E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1584,00 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

☒

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 1.7). 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	
Functie	
Plaats	
Datum	- -
Handtekening	

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix t 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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
1.3	Provide the title of the project.	Magnetothermal and current deep brain stimulation in experimental depression

2 Categories

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

- x☐ 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.

Neuromodulation is a new therapy in patients with severe neurological and psychiatric disorders [24]. In this context, deep brain stimulation (DBS) in patients with Parkinson's disease has become a routine therapy with beneficial effects at the short- and long-term. New insights in the neurobiology of depression suggest that DBS can be a potential

therapy in depression. During a trial of DBS of the ventral capsule/ventral striatum in obsessive-compulsive disorder, it was noted that patients' comorbid depressive symptoms also ameliorated [18]. This raised the question if DBS could be applied in treatment-resistant depression (TRD) solely. Recent research showed us that different open trials with DBS for treatment-resistant depression had positive results and a reduction in depression rating scales [17, 14, 20, 8, 5, 15, 9, 16, 13, 2, 21]. On the contrary, two randomized placebo-controlled trials, could not replicate these findings raising questions about the chosen study design, the best place of stimulation for TRD, the underlying activated microcircuits and the general mechanism of DBS [5].

Previous research identified a possible novel circuit involved in the neurobiology of mood disorders. This includes the ventromedial parts of the prefrontalcortex (vmPC), the subthalamic nucleus (STN), the substantia nigra reticularis, the nucleus accumbens, the lateral habenular nucleus (LHb) and the dorsal raphe nucleus (DRN) [11, 22, 1]. Previous results obtained in our laboratory, showed that in an animal model of mood disorder, DBS of the ventromedial part of the prefrontal cortex (vmPFC) had the most anti-depressant effects. Enhanced hedonia, reduced anxiety and a decreased forced-swim immobility are features seen after DBS of the vmPFC [12]. It would be interesting to gain further insight into the contribution of each specific region (infralimbic cortex, prelimbic cortex, and dorsal peduncular cortex) of the vmPFC and the underlying microcircuits involved. Until now there is a lack in understanding these precise underlying microcircuits and a more sophisticated understanding as to which specific regions play a key role in network dysfunction in these disorders is required.

Current DBS is the **golden standard** for neuromodulation, used in a variety of diseases. New research using this standardized method involves its application in discovering brain function and trying to modulate pathological conditions. However, one of the main disadvantages of current DBS is that it requires a fully implanted system, can cause damage to surrounding brain tissue, is a non-selective manner of stimulating cells and cannot be controlled or adjusted remotely. Besides unraveling the function of subregions of the vmPFC we are looking for a better alternative of neuromodulation that can overcome these disadvantages. In future research this might lead to a new more sophisticated technique of deep brain stimulation.

Very recently, the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA) has introduced a novel method of remote DBS using magnetic nanoparticles implanted in the brain. This is called 'magnetothermal DBS'. In this method, specific neurons are sensitized to heat by lentiviral delivery of heat-sensitive receptors (TRPV). These receptors can be activated by magnetic nanoparticles using an alternating magnetic field. This alternating magnetic field causes a fast vibration of the magnetic nanoparticles. These fast movements generate heat and activate the heat-sensitive receptors. Activation of these receptors cause neural excitation due to the influx of ions, which makes magnetothermal DBS a perfect method to remotely drive neural excitation [3]. This method of magnetothermal DBS can be driven more precisely than current DBS. This leads to a refined research strategy investigating precise stimulation of brain regions without implantation of electrodes. Here, we propose to apply this novel method of neuromodulation in well-validated models of experimental depression in animals [10].

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 aim of our project is to unravel a brain's microcircuit, involving specific populations of neurons in the vmPFC, limbic cortical areas, lateral habenular nucleus, subthalamic nucleus and midbrain 5-HT neurons, linked to mood-related behaviours by using a novel molecular and nanotechnology based neuromodulation approach. Since this method is still in its infancy we will perform these experiments alongside current DBS, the golden standard of neuromodulation, and compare their results.

We want to test the hypothesis that magnetothermal DBS of the vmPFC results in anti-depressant effects in an animal model of depression, and that this effect is linked to modulation of a circuit involving limbic cortical areas, the lateral habenular nucleus, the subthalamic nucleus and the midbrain 5-HT neurons.

We will do this by following three consecutive steps:

1. Evaluating which specific part of the vmPFC contributes to which specific antidepressant effects in a previously used animal model of depression.
2. Identification of neural microcircuits influenced by magnethothermal and current DBS of the vmPFC.
3. Analyzing neurochemical and electrophysiological changes of these microcircuits after magnetothermal and current DBS using microdialysis and electrophysiological recordings.

This approach may lead to the development of a better alternative of neuromodulation, remotely and wirelessly. It will help us uncover new mechanisms of brain function and in the future, with some adaptations of the technique, might function as a therapeutic strategy for patients with severe mood disorders like treatment-resistant depression.

The technique of current DBS is up and running in our lab so we can start straight away. The technique of magnetothermal DBS will be learned from the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). We will conduct behavioral experiments in the CUS model using experimental and control groups. We have experience with this animal model in our group. Identification of underlying microcircuits will be done with different immunohistochemical methods. Electrophysiological and neurochemical experiments will be learned and partially performed in the department of neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.).

3.3 Relevance

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

Major depression is one of the most common mental disorders carrying the heaviest burden of disability among mental and behavioral disorders [7]. Its life-time prevalence is estimated at 17% [19]. Depression can be treated using medication influencing neurotransmitter concentrations in the brain and psychotherapy. Despite extensive efforts to improve efficacy, still half of the patients remain fully or partially unresponsive to this therapy. Approximately 20% of these patients need more extreme treatment options including multiple antidepressants or electroconvulsive therapy (ECT) [16, 6]. For patients unresponsive to these therapies, a novel strategy called 'deep brain stimulation' (DBS) has made it entrance [4]. Although proven effective in different open-label trials, a recent randomized controlled trial did not show any positive results [5].

This indicates a lack in understanding the precise underlying microcircuits of this disorder. It shows that a more sophisticated understanding as to which specific regions play a key role in network dysfunction in this disorder and how is required. Further research and new techniques are needed to unravel these underlying neural microcircuits in mood disorders.

Magnetothermal DBS is a promising new therapy of neuromodulation. There is no need to insert electrodes as is done with current DBS and the area of activation and its parameters can be controlled more precisely. Its sensitivity and specificity is higher than with current DBS. With this new wireless and remote controlled technique we are able to modulate specific populations of neurons without severe damage to the brain.

Using this technique, we might unravel distinct microcircuits responsible for different aspects of mood. This will help us understand the mechanisms of mood disorders and might give us new target regions for neuromodulation. For human applications in the future, we can omit the usage of a lentivirus since heat-sensitive receptors (TRPV) are also endogenously expressed throughout the central nervous system. To enhance the neuromodulative signal, we artificially induce concentrations of this TRPV receptor. If we know which regions are of interest, we can adapt our technique only using endogenously expressed TRPV without utilization of a virus. This eventually might lead to a novel therapeutic strategy for patients with severe mood disorders.

3.4 Research strategy

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

The project will consist of several independent experiments which together will provide a multi-level, interdisciplinary investigation on which neural microcircuits and mood-related behaviours are influenced by current and magnetothermal DBS of the subregions of the vmPFC. It will show us what differs between magnetothermal and current DBS and if indeed magnetothermal DBS is a better alternative for neuromodulation than current DBS.

Our key objectives are:

- Identification of which part of the vmPFC (infralimbic cortex, prelimbic cortex, dorsal peduncular cortex) contributes to which specific antidepressant effects in an experimental depression model in animals.
- Identification of neural microcircuits involving specific populations of neurons in the limbic cortical areas, lateral habenular nucleus, subthalamic nucleus and midbrain 5-HT neurons influenced by magnetothermal and current DBS of the vmPFC.
- Identification of the underlying mechanisms linked to mood-related behaviours (ie. anhedonia, anxiety, food-motivation) influenced by magnetothermal and current DBS of the vmPFC.

First we will further dissect the function of vmPFC DBS in antidepressant behavior by individually stimulating the infralimbic cortex, prelimbic cortex, and dorsal peduncular cortex using current DBS in experimentally depressed rats (Fig.1). In this manner we can explore the contribution of each specific subregion of the vmPFC. We can investigate if stimulation of the different regions of the vmPFC changes different behavioral outcomes in different modalities of depression. In such a way we can personalize the stimulation paradigm in humans in the future based on their behavioral traits and the underlying mechanism responsible for these traits.

We will use an animal model of mood disorder: the chronic unpredictable stress (CUS) [12, 25] We have experience with this animal models in our group. This model covers multiple modalities of depressive behavior anxiety, behavioral despair, lack in motivation and hedonia. By stimulation different vmPFC regions we want to investigate if stimulation of that particular region in the vmPFC adapts a particular part of depressive-like behavior. Investigating this now might make it possible to individualize DBS treatment based on depressive behavioral traits later on.

Simultaneously we will conduct pilot studies of magnetothermal DBS in rats at the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA) (not in this protocol). Since this method for now is only working in anaesthetized mice we will have to extrapolate this technique to awake rats. During our exchange we will introduce this novel method in awake rats and stimulate our regions of interest.

Secondly we will conduct magnetothermal DBS experiments of the vmPFC (infralimbic, prelimbic and dorsal peduncular) in the same animal model of mood disorder (Fig.1). Neurons in the different parts of the vmPFC will be sensitized to heat by lentiviral delivery of heat sensitive receptor TRPV and injection of magnetic nanoparticles into these brain regions. Although the mammalian brain naturally expresses TRPV, we will use a transgene to gain sustained levels of TRPV. After sensitization we will be able to drive these neurons remotely using alternating magnetic fields. Upon alternating magnetic stimulation, the nanoparticles will dissipate heat via hysteretic power los. This will trigger activation of the TRPV receptor, the influx of ions and therefor activation of these TRPV positive neurons. We will evaluate and compare the effects of magnetothermal DBS and current DBS in all three regions of the vmPFC on mood- and movement-related behavioral parameters in the rodent models of experimental depression[12]. Also in this stimulation paradigm we will stimulate all three regions of the vmPFC.

In addition, we will perform a circuitry-investigation using immunohistochemical approaches, rodent PET-CT, electrophysiological and neurochemical investigations in order to understand the underlying mechanisms of magnetothermal and current DBS in an animal model of depression (Fig.1). With rodent PET-CT and immunohistochemical approaches we aim to discover activated brain regions during our experiments and therefore involved in the microcircuit of depressive-like behavior. Quantification of these potent brain regions will take place using post-mortem brain tissue and immunohistochemical staining. Only the most potent brain regions will undergo electrophysiological and neurochemical measurements. This means that the brain regions mostly activated during our animal models of depression and furthermore accessible for electrophysiology or microdialysis will undergo these

measurements. After electrophysiology or microdialysis all animals will be euthanized followed by transcardial perfusion to preserve the brain for post-mortem analyses.

The electrophysiological and neurochemical experiments will be learned and performed partially in the department of neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.) We will focus on regions known to be connected to the cortical area of interest. We hypothesize that these will include, the lateral habenular nucleus, the subthalamic nucleus, the ventral tegmental area and the 5-HT neurons in the forebrain containing the dorsal raphe nucleus [11, 23, 22, 1, 25].

In our opinion magnetothermal DBS can be a tremendous step forward for neuromodulation since we will be able to stimulate wirelessly, remotely and more precisely without an implanted system. With these experiments we can investigate responsible mechanisms for experimental depression in animals and test a better alternative of neuromodulation called 'magnetothermal DBS' that could overcome the disadvantages of current DBS.

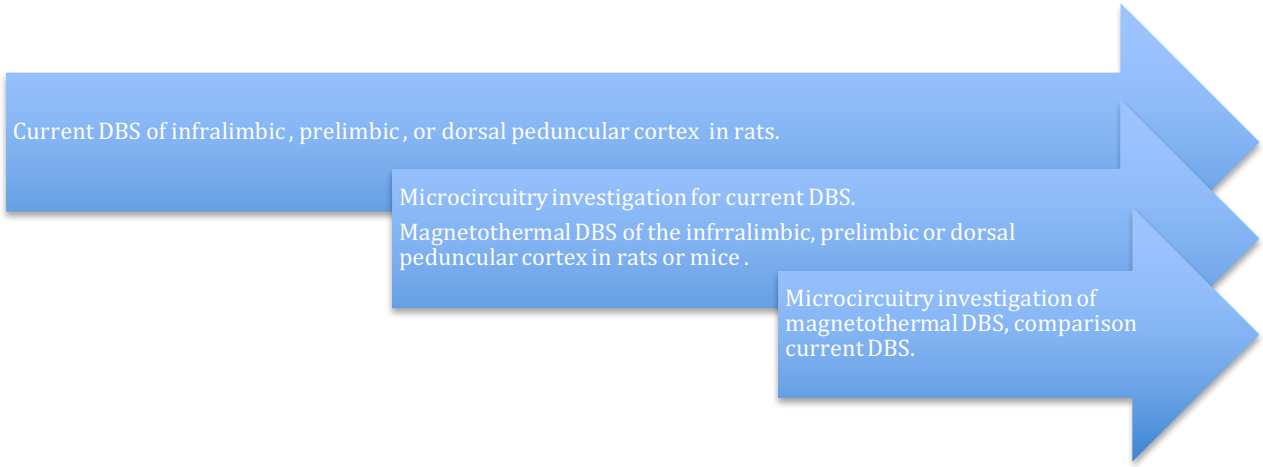

Fig. 1 **Time schedule of our 'magnetothermal and current deep brain stimulation in experimental depression' research project**

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.

Experiment I: **Current DBS in experimental depression**

i) 'Stimulation of the different regions of the vmPFC using current DBS'

We will stimulate the different regions of the vmPFC (infralimbic, prelimbic cortex, and dorsal peduncular cortex) using current DBS in rodent models of depression. We will use the chronic unpredictable stress (CUS) model. We will evaluate the effects of stimulation by comparing mood- and movement-related behavioral parameters and cognitive parameters between the different groups. We will identify the neural microcircuits influenced by current DBS of the different parts of the vmPFC using in vivo rodent PET-CT approaches, functional anatomical mapping, immediate early gene mapping and immunohistochemical stainings.

ii) ' Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression'

The most potent regions shown to be connected to the different parts of the vmPFC will undergo neurochemical or electrophysiological analysis upon stimulation. We will only investigate the most potent regions discovered during our previous experiments. We will perform microdialysis to measure extracellular levels of the main monoamines, before, during, and after current DBS[23]. We will determine changes in neuronal firing properties before, during and after current DBS using electrophysiological recordings. Both results will be compared to control groups.

We will learn electrophysiological and neurochemical experiments at the department of neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.) and execute them there and consecutively in our own laboratory.

Experiment II: **Magnetothermal DBS in experimental depression**

i) 'Stimulation of the different regions of the vmPFC using magnetothermal DBS'

We will test the hypothesis that magnetothermal DBS of the different parts of the vmPFC modifies different behavioral parameters linked to mood and cognitive parameters. To test this we will sensitize neurons in the three different parts of the vmPFC in rodents using lentiviral delivery of TRPV. We will activate these neurons using magnetothermal DBS. For this experiment we will use the chronic unpredictable stress (CUS) model to mimic experimental depression. In the end all rodents will undergo a battery of behavioral tests. We will evaluate the effects of magnetothermal DBS by comparing mood- and movement-related behavioral parameters and cognitive parameters between the different groups [4,2]. We will identify the neural microcircuits influenced by magnetothermal DBS of the different parts of the vmPFC using in vivo rodent PET-CT approaches, functional anatomical mapping, immediate early gene mapping and immunohistochemical stainings.

Magnetothermal DBS will be trained and refined in the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). Subsequently we will introduce this technique in our own laboratory and continue experiments here.

ii) 'Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression'

The most potent regions shown to be connected to the different parts of the vmPFC will undergo neurochemical or electrophysiological analysis upon stimulation. These brain regions will be obtained during our previous experiments. We will perform microdialysis to measure extracellular levels of the main monoamines, before, during, and after magnetothermal DBS[23]. We will determine changes in neuronal firing properties following magnetothermal DBS using electrophysiological recordings. Both results will be compared to control groups and the previous groups of current DBS.

The electrophysiological and neurochemical experiments will be learned and firstly performed at the department of neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.) Consecutively we will perform these experiments in our own laboratory.

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.

Our project will consist of different inter-related sections, which together will provide a multi-level, interdisciplinary investigation on how magnetothermal DBS of different parts of the vmPFC influences different aspects of mood, mood- and movement-related behavior and cognitive parameters. It will enable

us to identify and modulate microcircuits involved in different aspects of mood. Furthermore, it will enable us to compare magnetothermal DBS to current DBS to see what differs between these two methods and to investigate if indeed magnetothermal DBS is a better alternative for neuromodulation than current DBS. We can also use the magnetothermal DBS to fine-tune the stimulation parameters of current DBS.

We will do this by:
1. Evaluating which specific part of the vmPFC contributes to which specific antidepressant effects in a previously used rodent model of depression. We will subdivide the vmPFC into its infralimbic, prelimbic and dorsal peduncular region stimulating them separately with current DBS and magnetothermal DBS. For this evaluation we will use a battery of mood- and movement-related behavioral tasks and cognitive tasks.
2. Identification of neural microcircuits influenced by magnethothermal and current DBS of the different parts of the vmPFC. We will investigate activated microcircuits in post-mortem brain tissue obtained from our first experiment. We will use different imaging strategies including immunohistochemical stainings.
3. Analyzing neurochemical and electrophysiological changes of the most potent activated brain regions during magnetothermal and current DBS using microdialysis and electrophysiological recordings. These brain regions involved in the underlying microcircuits of depression will be identified during our previous experiments.

Depression covers multiple modalities including anxiety, behavioral despair, lack in motivation, hedonia and cognition. Traits of the disease vary from patient to patient. We want to discover if stimulation of different regions of the vmPFC changes different behavioral outcomes in different modalities. In this way we can investigate the underlying mechanism responsible for these traits and possibly personalize the stimulation paradigm in humans in the future based on their behavioral traits. For this reason, we need to stimulate all three regions of the vmPFC.

Magnetothermal DBS is a novel technique of neuromodulation that is more selective and more refined than current DBS. So far magnetothermal DBS has been applied in anaesthetized mice. We want to conduct experiments in awake rats therefore we will collaborate with the department of Materials Science and Engineering at MIT (Boston, USA) adapting this novel technique to our preferred settings. We want to extrapolate this model to rats since we have more expertise in using rats for deep brain stimulation experiments.

Magnetothermal DBS can be controlled remotely and wirelessly making it a promising technique for future applications. It will be a better alternative than current DBS, which for now is the golden standard. In our research we will compare this novel technique with current DBS to investigate if magnetothermal DBS is indeed a better stimulation paradigm and to identify and compare the underlying microcircuits responsible for depressive-like behavior. We can also use the information obtained from magnetothermal DBS to fine-tune stimulation parameters used for current DBS.

This approach will help us uncover new mechanism of brain function in experimental-depression and in the future may lead to the development of a novel therapeutic strategy for patients with severe mood disorders like refractory depression.

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	Stimulation of the different regions of the vmPFC using current DBS
2	Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression using current DBS
3	Stimulation of the different regions of the vmPFC using magnetothermal DBS
4	Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression using magnetothermal DBS
5	
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Centrale Commissie Dierproeven

16.

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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University

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
1	Stimulation of the different regions of the ventromedial prefrontal cortex (vmPFC) using current DBS

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 experimental approach of this study is to identify which specific part of the ventromedial prefrontal cortex (vmPFC) causes which specific antidepressant effects in an animal model of depression. We will include sham control groups not receiving actual stimulation and sham control groups not experiencing stress from the CUS animal model.

Firstly, we will insert electrodes for deep brain stimulation (DBS) under general anaesthesia in experimental and sham control animals. Electrodes will be placed in either in the infralimbic, prelimbic or dorsal peduncular cortex in the vmPFC using a stereotactic frame. We will only stimulate in the experimental groups.

Secondly, to gain depressive-like behaviour in our animals we will use the chronic unpredictable stress (CUS) model. For the CUS model, all animals will be exposed to a certain period of stress.

After a period of chronic stress, all animals will undergo behavioural testing. The maximal duration of stress including behavioural testing will be ten weeks. The animals in the experimental group will undergo current DBS during these behavioural tasks. Electrodes of the sham control group will be connected to the stimulator but no stimulation will be given. We will test antidepressant effects of DBS of different regions in the vmPFC by comparing behavioural outcomes between experimental and sham

control groups.

Consecutively we want to identify the underlying neural microcircuits activated by current DBS. We will visualize modulated brain regions and recent activated neurons by using in vivo rodent PET-CT approaches, post-mortem functional anatomical mapping, post-mortem immediate early gene mapping and post-mortem immunohistochemical stainings. After PET-CT we will perfuse all animals and fixate their brains for post-mortem analysis.

PET-CT alone will not be enough to distinct specific brain regions since the spatial resolution is low and it is not possible to specify different types of cells. This will only be possible using post mortem brain analysis. For this reason, animals cannot function as their own control and a different sham control group is needed. We will perform PET-CT on the first animals tested to serve as a pilot study. If during this pilot, spatial resolution of PET-CT turns out to be too low for good discrimination between areas of interest we will only use post-mortem brain analysis.

During brain analysis we will include scanning the lateral habenular nucleus (LHb), the subthalamic nucleus (STN), the ventral tegmental area and the 5-HT neurons in the midbrain containing the dorsal raphe nucleus (DRN) since previous research has shown us that these regions are of particular interest [6, 9, 8, 2].

The primary outcome:

- Mood- and movement related behavioural parameters tested with a battery of behavioural tasks. We will compare outcomes between the experimental and control groups.

Secondary outcomes:

- PET-CT images and their alterations between experimental and control groups.
- Neuronal alterations between experimental and control groups using post-mortem immunohistochemical analysis. We will use various staining techniques (i.e. immediate early gene mapping, functional anatomical mapping, cell type and neurotransmitter specification) and quantify:
  i) Changes in recent neuronal activity
  ii) Changes in the number of neurons expressing different neurotransmitters
  iii) Changes in the distribution of GABAergic, glutamatergic and dopaminergic markers.
  We will scan multiple potent brain regions possibly involved in a microcircuit following DBS of the vmPFC.

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

Surgery

All animals will undergo surgery where we stereotactically implant a bilateral stimulating electrode in different regions of the vmPFC (infralimbic, prelimbic, dorsal peduncular cortex). Surgery will be performed under general anaesthesia. After implantation all animals will get a post-operative recovery period.

Animal models

For our experiments we will use the CUS animal model. This model has been proven effective in mice and rats and is a well-validated animal models for experimental stress and depression [4, 7]. For the CUS model we will vary multiple unpredictable stressors during 3 consecutive weeks. These stressors will include a shift in hours of light per day, exposure to stroboscopic light (preferably 2.5 Hz), tilt cages, soiled bedding, paired housing and so on. During paired-housing, animals will be grouped in pairs with different partners. All stressors will last for 10-14 hours and since they should be unpredictable the exact application times are not fixed. The stressors will always take place during the active period of the animal in a reversed day-night cycle in the laboratory. One will always take place in the morning while the other stressor will always take place in the afternoon or evening. We will continue these stressors during our DBS experiments and behavioural testing, but always apply these stressors after behavioural testing. The maximal duration of stressor exposure will be ten weeks. During this model, all animals will be monitored

daily.

For this appendix we will start with rats since previous research in our laboratory, showing antidepressant results of vmPFC DBS, was also conducted in rats [6]. This part of our research is linked to these previous conducted experiments. We have more experience with deep brain stimulation and behavioral experiments in rats and stimulating the right brain regions in rats is easier because of their bigger brain size.

We are interested in the underlying mechanism of depression and its microcircuits and the CUS model is the appropriate model to investigate our research questions. After the previously described period of stress, all animals will undergo a battery of behavioural tasks with or without current DBS.

Behavioural testing
We will evaluate the effects of current DBS of the vmPFC by measuring different behavioral parameters linked to mood and cognition. During the behavioral tasks, animals will receive either stimulation or sham stimulation (cables connected but stimulator off.) We will conduct behavioral tasks focused on anhedonia, anxiety, motivation, behavioral despair and cognition [7, 12, 3, 8]. A summary of the different behavioral tests and their readout parameters is listed below. The selection of tests may change in the course of the study due to analyses of previous tests or for logistic reasons.

Table 1. Summary of the different behavioural tests, readout parameters and discomfort.

Test paradigm	Item(s)	Readout parameter(s)	Discomfort
Forced swim task	mood	immobility duration, latency to immobility, swimming duration, climbing duration.	Moderate discomfort, animals will be in water
Sucrose intake/preference	mood	volume of sucrose intake corrected for animal weight.	Mild discomfort, results in increased anxiety
Food intake	mood	volume of food intake corrected for animal weight.	Moderate discomfort, animals will receive restricted feeding regimes or deprivation
Open field	anxiety and locomotion	total distance travelled, average speed and time spent in corners.	Mild discomfort, results in increased anxiety
Elevated zero maze	anxiety	time spent in closed arms	Mild discomfort, results in increased anxiety
Home-cage emergency	impulsivity and anxiety	latency to escape	Mild discomfort, results in increased anxiety
Barnes maze	memory	number of errors per trial, rate of decline in number of errors and path length.	Mild discomfort, results in increased anxiety
Morris water maze	memory	time spent in the platform quadrant, escape latency and swimming speed.	Moderate discomfort, animals will be in water
Cued and contextual fear conditioning	memory	percentage of time spent freezing and time to extinction.	Moderate discomfort, small electrical shock will be administered
Object location task	memory	ratio of time spent at object in new versus old location.	Mild discomfort, results in increased anxiety
Object recognition task	memory	ratio of time spent at new versus old object.	Mild discomfort, results in increased

			anxiety
Y-maze	memory	Ratio left-right discrimination	Mild discomfort, results in increased anxiety
Skinner box paradigms e.g. reaction time and attention tasks	cognition	reaction time, incorrect responses, correct responses, premature responses	Moderate discomfort, animals will receive restricted feeding regimes or deprivation

The maximum number of behavioural tests is 7. We expect further reduction of the number of behavioural tests during our research project. Every single test describes a different behavioural feature and since we will test anti-depressive behaviour we will need various modalities to test and compare. Depression can even interfere with cognition; therefore we also include behavioral experiments testing this modality [3]. Behavioural tests that show a significant difference between our experimental and control groups will be used in consecutive studies. It might occur that multiple behavioural tests are suitable for our research question of interest. We will then select the behavioural task with the least degree of discomfort. Cumulative discomfort caused by consequent behavioural testing will be minimized by a wash out period between the different tests.

PET-CT and immunohistochemistry
After behavioural testing animals will be anesthetized to perform an in vivo rodent PET-CT and will be overdosed with pentobarbital followed by consecutive transcardial perfusion to isolate en preserved their brain for further analysis. We will conduct PET-CT pilots on our first animals to identify if the spatial resolution is good enough to discriminate between interesting brain regions. We will perform post-mortem functional anatomical mapping, immediate early gene mapping (ie. C-fos) and immunohistochemical staining's. These techniques enable us to quantify:

i) Changes in recent neuronal activity after DBS.
ii) Changes in the number of neurons expressing different neurotransmitters after DBS.
iii) Changes in the distribution of GABAergic, glutamatergic and dopaminergic markers after DBS.

We will scan multiple potent brain regions possibly involved in a microcircuit following current DBS of the vmPFC. All methods are widely used in our research group. With these techniques we will be able to identify which brain regions are activated after current DBS of different parts of the vmPFC and to which neurons they are connected.

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 needed, we considered published studies and other previous studies by our group [7]. To minimize the number of animals used, we will perform a power analyses. In each group we will perfuse all animals and fixate their brains for post-mortem analysis. This is needed to specify neuronal changes between experimental and control groups. For this reason animals cannot function as their own control and a different sham control group is needed.

B. The animals

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

Species

We will use adult male Sprague Dawley rats purchased from a registered breeding facility. We will use this specie because they can be used in the CUS model and because our research group is trained with DBS surgery in rats.

Gender

We will use only male rats since previous research has shown that the estrogen cycle of female rodents can interfere with behavior and the release of neurotransmitters [1, 10]. Their estrogen cycle will cause a larger spread in behavioral outcomes, which will interfere with our readout measurements [10].

Number of animals

The estimation of the number of animals needed per group is based on previous research done in our research group [7]. In their experiments they tested behavioral anti-depressant effects following electrical stimulation of the vmPFC in the CUS animal model. They used a maximum of 15 animals per group. In our experiments we will use the same animal model and stimulate subregions in the same brain area. Therefore, we will most likely use a maximum of 15 animals per group as well. We will have three different stimulation groups (infralimbic cortex, prelimbic cortex and dorsal peduncular cortex), their sham control groups and naïve control groups.

Before starting experiments, we will perform a power analysis and take into account a possible drop out of animals. Animal dropout during our experiments can be due to: surgical complications, electrode loss, incorrect electrode localization or premature termination of the experiments due to too much stress during the CUS model. Within our research group the dropout of animals' ranges from 10-25% depending on the specific surgical procedures, the behavioral paradigm and the given tasks. We will take into account a maximum dropout of 25% and aim to reduce this number during the research project.

For this appendix we will start with rats since previous research in our laboratory, showing antidepressant results of vmPFC DBS, was also conducted in rats [6]. This part of our research is linked to these previous conducted experiments

We will use 'depressed' animals with the CUS model and 'non-depressed' animals to function as their control. Each group will consist of a subgroup receiving current DBS and a subgroup functioning as sham control. We will implant the electrodes in three different subregions of the vmPFC. We will use a maximal amount of 15 animals per group. This will lead to: the experimental group CUS+ with current DBS (infralimbic=15, prelimbic=15, dorsal peduncular=15; n=45) and their sham controls (n=10). And the naïve group CUS- with current DBS (n=45) and their sham controls (n=10). Taken together this will include 110 animals (Fig. 1). Furthermore a possible 25% dropout needs to be taken into consideration. Taken all this into account we will use a maximal amount of 138 rats.

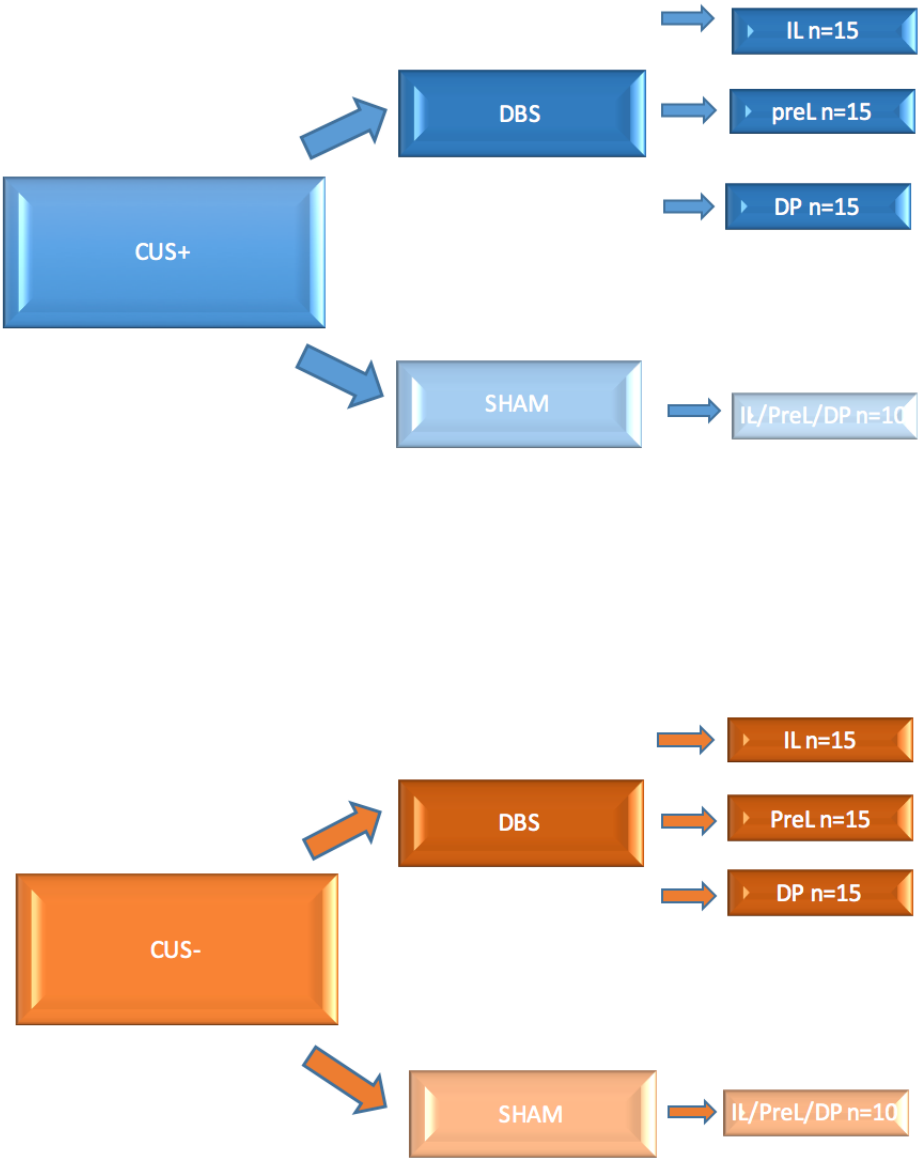

Fig. 1. Subdivision of groups for Current DBS.

C. Re-use

Will the animals be re-used?

x☐ 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.

Replacement
The primary aim of this study is to identify which part of the vmPFC gives which antidepressant effects and to identify their neural underlying microcircuits. This cannot be achieved using in vitro experiments or in computer models since we need a whole complex neural network inducing behavior. The loss of complexity in neural networks using lower animal species will generate results not easily translated to humans. This study can also not be performed in humans since we need post-mortem analysis of the brain for identification of the microcircuits linked to stimulation of the vmPFC.

Reduction
We will minimize the number of animals in this study by using a power analysis. Further reduction will take place by combining PET-CT approaches with anatomical mapping, immediate early gene expression and immunohistochemical staining's. We have limited the estimated number of animals needed per group based on previous results of our research group where stimulation of the vmPFC in rats leads to a reduction in depressive-like behavior [7].

Refinement
Current DBS is widely used in our lab so the technique is already refined. To refine the CUS model we will be trained by previous researchers in our group and work together with the psychiatry department whom also has experience using this model. We will use general anesthesia during insertion of the current DBS electrodes. All animals will receive appropriate analgesics during their post-operative recovery period when needed. PET-CT and various immunohistochemical stainings are widely used in our lab so less error in these techniques is expected. We will anaesthetize all animals before PET-CT followed by an overdose of pentobarbital and consecutive transcardial perfusion. Both reduces animal discomfort to a minimum.

With this chronic stress model we have the ability to simultaneously produce a set of behavioral alterations together with strong face validity**.** The model is based on clinical evidence that the risk of depressive episodes increases after multiple chronic stressful life events [4]. To mimic depression this models is the best we can use which is necessary for reaching our objectives. Too further refine our experiment we will insert different human endpoints.

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

All animals will be under general anaesthesia during insertion of current DBS electrodes and will receive appropriate analgesics during the post-operative recovery period.
After the behavioral experiments all animals will be anaesthetized and undergo PET-CT followed by an overdose pentobarbital and consecutive transcardial perfusion. In this way discomfort is kept to a minimum. To minimize suffering during our experiments we will insert human endpoints in our protocol as described below.

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.

It is just recently discovered that current DBS of the vmPFC causes antidepressant effects in an experimental depression model in rats [7]. Since we will subdivide this region into the infralimbic, prelimbic and dorsal penduncular cortex we will be the first to further investigate which specific region is responsible for this effect. No articles concerning this topic has been published so far. We will need this to discover the underlying microcircuits upon stimulation of the different prefrontal areas, to discover if particular behavioral traits can be assigned to stimulation of particular prefrontal areas.
Taken all together, this wil give a better understanding in the microcircuits involved in depressive-like

behavior in experimental depresion.

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

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

All animals will be housed individually.

For the chronic unpredictable stress (CUS) model all animals will undergo variable stressors for the maximal duration of ten weeks. We will vary the stressors daily to induce chronic stress, which is the basis of our research. For this animal model indivual housing is nescesarry since it enhances susceptiblility to these chronic stressors [11].

We will integrate the insertion of DBS electrodes in this animal model of experimental depression.

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?

x☐ 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.

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

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

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

We will use general anesthesia during stereotactic insertion of the DBS electrodes in the brain. All animals will receive appropriate analgesics during the post-operative recovery period when needed. Animals will be monitored daily during the experimental procedures stated in section 2A and will be checked for signs of pain or discomfort. PET-CT will be performed under general anesthesia and consecutive transcardial perfusion after an overdose of pentobarbital. Both will reduce animal discomfort to a minimum. To minimalize pain or suffering we will insert human endpoints in our protocol as described below.

I. Other aspects compromising the welfare of the animals

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

Animals will experience chronic stress by adding variable stressors during our experimental model of depression. Our research is based on experimental depression and the previous mentioned validated animal model for experimental depression includes exposure to chronic stress.

Animals will experience additional stress during the battery of behavioural tasks.

Another adverse effect of stereotactic insertion of the current DBS electrodes could be loss of an electrode construct.

Explain why these effects may emerge.

Stress during our experimental depression animal model will occur since we will apply daily stressors such as; tilt cages, soiled bedding or stroboscopic light.

Additional stress will emerge when executing particular behavioural tasks such as the forced swim task.

Loss of an electrode construct might be due to scratching of these electrodes.

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

During our CUS model, all animals will be inspected daily. When an animal shows too much stress, i.e. a disrupted physical appearance together with disrupted unprovoked behaviour and or abnormal responses to external stimuli and or self-induced trauma, we will remove it from the experiment. This will be discussed in more detail in our humane endpoints and working protocol.

Cumulative discomfort caused by consequent behavioural testing will be minimized by a wash out period between the different tests.

To prevent loss of the electrodes we will secure the electrodes to the skull in position using dental cement. We will give all animals a recovery-period that allows the skin around the electrodes to recover. During our recovery period and experiment, all animals will be inspected daily.

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.

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

Human endpoints will mainly be based on general considerations regarding general health, too much discomfort in the CUS animal model or complications of stereotactic surgery.

Human endpoints will include:

-Signs of a disturbed physical appearance, disturbed unprovoked behavior, self-induced trauma or moderately abnormal responses to external stimuli.

-Rapid or progressive weight loss together with a loss of body condition compared to the beginning of our experiments. During our experiment we will inspect all animals daily using the general impression and physiological condition score format. We will weight all animals several times a week. Only measuring weight changes or disturbances in body condition is not a good way to interpret general health and therefore we will use both measurements scales together[5].

-Signs of untreatable local or generalized infection for more than 3 days.

If a human endpoint is reached we will take the animal out of the experiment, euthanize them with an overdose of pentobarbital followed by transcardial perfusion for post-mortem analysis. At the end of the experiments, all animals will be sacrificed using an overdose of pentobarbital followed by transcardial perfusion for post-mortem brain analysis.

Indicate the likely incidence.

We estimate the likely incidence at maximally 25%, for this reason we included this percentage of dropouts in our maximal estimated number of animals.

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').

All animals will undergo moderate discomfort following surgery.
75% will undergo severe discomfort during the induced depression and following experiments while 25% of all animals will undergo mild discomfort as they serve as non-depressed controls.

Surgery:
The level of discomfort following stereotactic insertion of current DBS electrodes will be moderate.

CUS animal model:
The expected level of discomfort due to the CUS animal model with individual housing is severe. This animal model of experimental depression will give a high level of stress during several consecutive weeks.

Deep brain stimulation:
The level of discomfort following DBS will be mild.

Behavioural testing:
The level of discomfort during our behavioural testing will be mild to moderate (table 1). Cumulative discomfort caused by consequent behavioural testing will be minimized by a wash out period between the different tests. At the end of these experiments, all animals will be sacrificed with an overdose of pentobarbital followed by transcardial perfusion for post mortem brain analysis.

Euthanasie:
At the end of our experiments all animals will undergo general anaesthesia, PET-CT and will get an overdose pentobarbital followed by transcardial perfusion for brain preservation. These procedures will give mild discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

All animals will be sacrificed at the end of the experiments to isolate their brain and perform functional anatomical mapping, immediate early gene mapping and immunohistochemical staining's.

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.

x☐ Yes

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3. Henningsen, K., J.T. Andreasen, E.V. Bouzinova, M.N. Jayatissa, M.S. Jensen, J.P. Redrobe, and O. Wiborg, *Cognitive deficits in the rat chronic mild stress model for depression: relation to anhedonic-like responses.* Behav Brain Res, 2009. **198**(1): p. 136-41.
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6. Lim, L.W., M.L. Janssen, E. Kocabicak, and Y. Temel, *The antidepressant effects of ventromedial prefrontal cortex stimulation is associated with neural activation in the medial part of the subthalamic nucleus.* Behav Brain Res, 2015. **279**: p. 17-21.
7. Lim, L.W., J. Prickaerts, G. Huguet, E. Kadar, H. Hartung, T. Sharp, and Y. Temel, *Electrical stimulation alleviates depressive-like behaviors of rats: investigation of brain targets and potential mechanisms.* Transl Psychiatry, 2015. **5**: p. e535.
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10. van Goethem, N.P., K. Rutten, F.J. van der Staay, L.A. Jans, S. Akkerman, H.W. Steinbusch, etal.J. Prickaerts, *Object recognition testing: rodent species, strains, housing conditions, and estrous cycle.* Behav Brain Res, 2012. **232**(2): p. 323-34.
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12. Yang, L.M., B. Hu, Y.H. Xia, B.L. Zhang, and H. Zhao, *Lateral habenula lesions improve the behavioral response in depressed rats via increasing the serotonin level in dorsal raphe nucleus.* Behav Brain Res, 2008. **188**(1): p. 84-90.

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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University

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
2	Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression using current DBS

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 experimental approach of this study is to measure changes in extracellular levels of the main monoamines and changes in electrophysiological properties in potent brain regions during current DBS of the different parts of the vmPFC. In our first experiment we aim to discover activated brain regions during our stress protocol and DBS of the vmPFC, therefore involved in the microcircuit of depressive-like behavior. Quantification of these potent brain regions will take place using post-mortem brain tissue of our first experimental group (appendix 1). Only the brain regions mostly activated during our animal models of depression and furthermore accessible for electrophysiology and microdialysis will undergo these measurements during this experiment. We will include sham control groups not receiving actual stimulation and sham control groups not experiencing stress from the CUS animal model.

Similar to the study described in appendix 1, we will firstly implant DBS electrodes in different regions of the vmPFC using a stereotactic frame. Electrodes will be placed in either the infralimbic, prelimbic or dorsal penduncular cortex of the vmPFC. Additionally, in one part of the animals, we will insert a cannula in a brain region of interest that is involved in the microcircuits of depressive-like behaviour in our animal model. We will have different experimental groups targeting a different brain region for future microdialysis. Which regions are of interest is to be determined in our previous experiment. This cannula will serve as an access point for microdialysis in freely moving rats during DBS. We will also add sham control groups. The other part of animals will nog undergo microdialysis but electrophysiological measurements.

We will gain depressive-like behaviour in animals using the chronic unpredictable stress (CUS) model.

In our first experiment, stated in appendix 1, we have gained insight into the underlying microcircuits activated by DBS of the vmPFC. In this experiment we want to investigate what changes in the most potent regions of the microcircuit found in our previous experiment. We hypothesize that the lateral habenular nucleus (LHb), the subthalamic nucleus (STN), and the dorsal raphe nucleus (DRN) will be regions of interest [6, 11, 10, 2].

During DBS, the microdialysis probes will be guided through the cannula and intracranial sampling of neurotransmitters will be performed. Measurements will take place before, during and after stimulation. The other part of the animals will undergo electrophysiological recordings different regions of interest under general anesthesia. For all animals either microdialysis or electrophysiological measurements will be their final experiment followed by an overdose of pentobarbital and transcardial perfusion to preserve the brain for post-mortem analyses. We will measure shortly before, during and shortly after DBS. Which regions are of interest is to be determined in our previous experiment explained in appendix 1.

The primary outcomes:

- Changes in monoamine levels in specific brain regions upon DBS of the different parts of the vmPFC.
- Changes in electrophysiological properties in specific brain regions upon DBS of the different parts of the vmPFC.

  We will investigate the most potent brain regions involved in the underlying microcircuit following DBS of the vmPFC observed in our previous experiment. This means that the brain regions mostly activated during our animal model of depression and furthermore accessible for electrophysiology or microdialysis will undergo these measurements. We will measure before, during and after deep brain stimulation.

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

Surgery
All animals will undergo surgery where we stereotactically implant a bilateral stimulating electrode in different regions of the vmPFC (infralimbic, prelimbic, dorsal peduncular cortex). A part of these animals will be implanted with a cannula for microdialysis. A part of the animals will not be implanted with a cannula and will undergo electrophysiology instead of microdialysis. Surgery will be performed under general anaesthesia. After implantation all animals will get a two-week recovery period.

Animal models
For our experiments we will use the CUS model. This model has been proven effective in mice and rats and is a well-validated animal models for experimental stress and depression [4, 7]. For the CUS model we will vary multiple unpredictable stressors during 3 consecutive weeks. These stressors will include a shift in hours of light per day, exposure to stroboscopic light (preferably 2.5 Hz), tilt cages, soiled bedding, paired housing and so on. During paired-housing, animals will be grouped in pairs with different partners. All stressors will last for 10-14 hours and since they should be unpredictable the exact application times are not fixed. They will take place during their active period of the animal in a reversed day-night cycle in our laboratory. One will always take place in the morning while the other stressor will always take place in the afternoon or evening. We will continue these stressors during our DBS experiments. The maximal duration of stressor exposure will be ten weeks; including the days of experimental microdialysis or electrophysiology.

We are interested in the underlying mechanism of depression and its microcircuits; therefore, the CUS model is the appropriate models to investigate our research questions.

Microdialysis and electrophysiology
After a period of stress all animals will undergo current DBS of different parts of the vmPFC, microdialysis or electrophysiological recordings of different parts of the microcircuits discovered in our previous research. The maximal duration of stress will be ten weeks. Before, during and after DBS in freely moving animals we will collect CSF samples via the cannula implanted during surgery. In these samples we will analyse different monoamine concentrations to discover what changes during DBS of different sub regions of the vmPFC. Animals will be sacrificed after microdialysis using an overdose of pentobarbital followed by transcardial perfusion. Part of the animals will undergo general anaesthesia for electrophysiological recordings of other potent brain regions found in our previous experiment. We will record shortly before, during and shortly after DBS.

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 needed, we considered published studies and other previous studies by our group [7]. To minimize the number of animals used, we will perform a power analyses. In each group we will perfuse all animals and fixate their brains for post-mortem analysis. This is needed to specify neuronal changes between experimental and control groups later on. For this reason animals cannot function as their own control and a different sham control group is needed.

B. The animals

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

Species

We will use adult male Sprague Dawley rats purchased from a registered breeding facility. We will use these animals since we have experience with them in our group, because they can be used in the CUS model and because microdialysis and electrophysiology can easily be performed on these animals [7, 8, 11, 3]. Since magnetothermal DBS is now working in anaesthetized mice, we need to extrapolate this method to rats. We are currently conducting pilot studies of magnetothermal DBS in awake rats in the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA) (not in this protocol).

Gender

We will use only male rats since previous research has shown that the estrogen cycle of female rodents can interfere with behavior and the release of neurotransmitters [1, 12]. Their estrogen cycle will cause a larger spread in behavioral outcomes, which will interfere with our readout measurements [12].

Number of animals

The estimation of the number of animals needed per group is based on previous research done in our research group [7, 11]. In one of the mentioned experiments they tested behavioral anti-depressant effects following electrical stimulation of the vmPFC in the CUS animal model. In another experiment they performed microdialysis and electrophysiological recordings in rats. Both researchers used a maximum of 15 animals per group. Microdialysis of the vmPFC in mice has been performed as well [9]. They used a number of 12 animals per group. In our experiments we will also use the CUS animal model, stimulate subregions of the same vmPFC and perform microdialysis and electrophysiological recordings as well. Therefore, we will use approximately the same amount of animals per group with a maximum of 15 rats per group.

Before starting experiments, we will perform a power analysis and take into account a possible drop out of animals. Animal dropout during our experiments can be due to: surgical complications, electrode loss, incorrect electrode localization, cannula loss, too much stress during the CUS model or complications during electrophysiology. We will take into account a dropout of 25% and aim to

reduce this number during the research project.

We will stimulate infralimbic, prelimbic or dorsal peduncular cortex while measuring one potent brain region using microdialysis and if possible one region with electrophysiology. How many brain regions will undergo microdialysis or electrophysiological measurements is to be determined in our first experiment. We will explore a maximum of three potent brain regions. If only one or two regions are of interest after our previous experiments including immunohistochemistry, we will only test these brain regions. In 1 animal we will insert 1 cannula for microdialysis. For electrophysiological measurements we can try to measure different regions of interest within 1 animal but for now we take one brain region into account. Electrophysiology will be under general anesthesia so measuring more regions will not increase the severity of discomfort.

We will use 'depressed' animals with the CUS model and 'non-depressed' animals to function as their control. Each group will consist of a subgroup receiving current DBS and a subgroup functioning as sham control. We will implant the electrodes in three different subregions of the vmPFC. We will use a maximal amount of 10 animals per group. We will explore a maximum amount of three potent brain regions for microdialysis and electrophysiology.

This will lead to:

Microdialysis: the experimental group CUS+ with current DBS (infralimbic, prelimbic, dorsal peduncular; n=45) in three potent brain regions (n=135), and their sham controls (n=30). And the naïve group CUS- with current DBS (n=135) and their sham controls (n=30) (Figure 1.).

We will start with an animal batch of n=8 per group for microdialysis. If this group size already gives significant differences, we will leave the group size at n=8 in total and do no more microdialysis experiments. The sham control group will then be n=5. This will reduce the amount of animals to n=174 for microdialysis.

Electrophysiology: the experimental group CUS+ with current DBS (infralimbic, prelimbic, dorsal peduncular; n=45) in three potent brain regions (n=135), and their sham controls (n=30). And the naïve group CUS- with current DBS (n=135) and their sham controls (n=30) (Fig. 1).

We will start with an animal batch of n=8 per group for electrophysiology. We need a particular amount of neurons for electrophysiology to measure electrophysiological differences upon stimulation between the different groups. Previous research has shown electrophysiological effects in the dorsal raphe nucleus upon vmPFC stimulation, using n=18 neurons [7]. We therefore will use a minimum of n=18 neurons. If eight animals per group will give enough neurons for statistical analysis, we will only test eight animals per group. The sham control group will then be n=5. This will reduce the amount of animals to n=174 for electrophysiology.

Furthermore, a possible 25% dropout needs to be taken into consideration.

Taken all this into account we will use n=435 animals, when n=8 per experimental group and n=5 per sham group already give measurable outcomes for microdialysis and enough neurons for electrophysiological measurements. If this is not the case, we will introduce another animal batch per group resulting in the maximum amount of 825 rats.

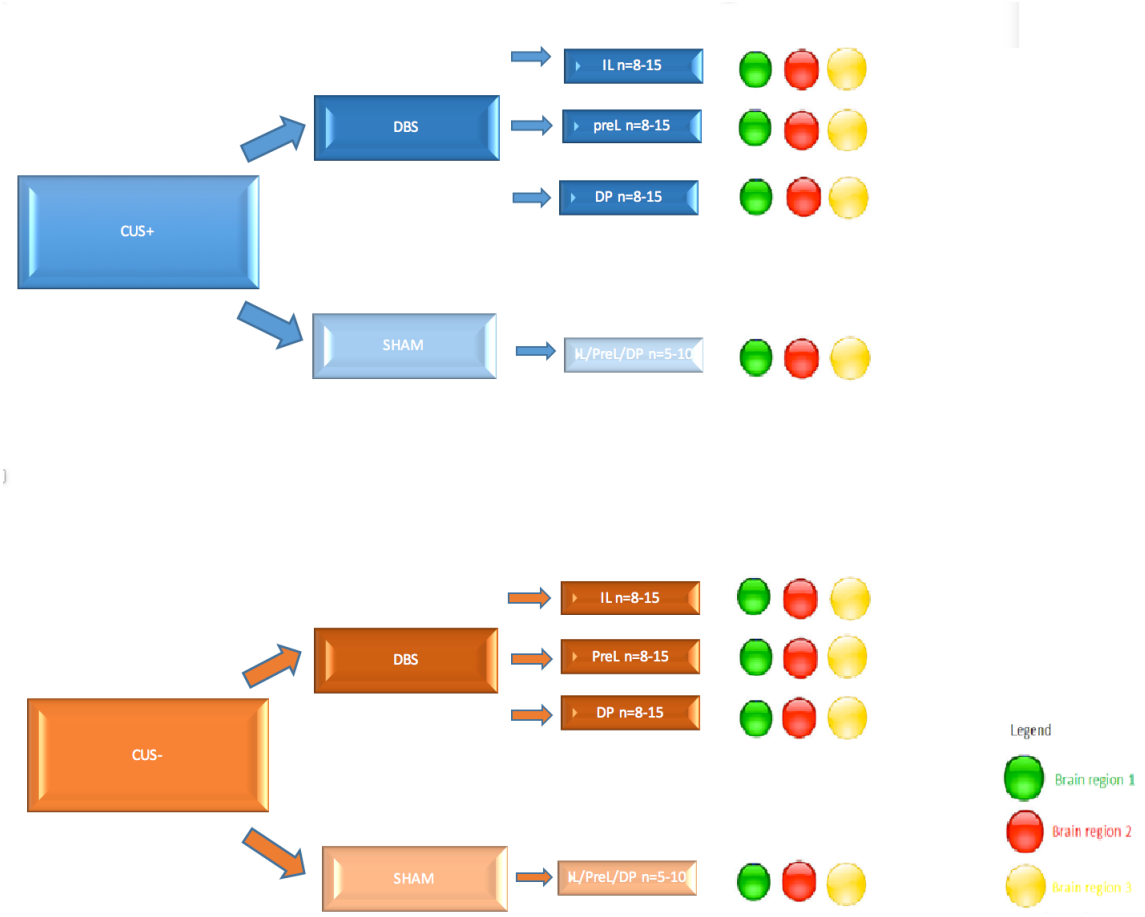

Fig. 1. Subdivision of groups for Current DBS and additional microdialysis (n=8-15) or electrophysiology (n=8-15), together with a sham control group (n=5-10). Each brain region indicated with a green, red or yellow circle, will either be a microdialysis experiment or an electrophysiological experiment.

C. Re-use

Will the animals be re-used?

x☐ 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.

Replacement
The primary aim of this study is to investigate the effect of infralimbic cortex, prelimbic cortex and dorsal peduncular cortex current DBS on the levels of monoamines and electrophysiological properties in different forebrain regions. This will help us to uncover the underlying mechanisms of

antidepressant effects of current DBS in the vmPFC. This cannot be achieved using in vitro experiments or in computer models since we need a complex 'depressed' neural microcircuit to detect these changes. The loss of complexity in neural networks using lower animal species will generate results not easily translated to humans. This study cannot be performed in humans since this micro circuitry investigation using microdialysis and electrophysiology will not be ethically approved.

Reduction
We have chosen a validated and well-known model of experimental stress and depression used before in our laboratory. In this way we limit the number of animals in our study since differences in specific behavioural-parameters following current DBS has been shown before. We will hypothesize that also underlying changes in monoamines and electrophysiological properties can be detected in these models. We have limited the number of animals based on previous results of the influence of current DBS in the vmPFC and microdialysis together with electrophysiological measurements in different brain regions [7, 11]. To further reduce the amount of animals we will start with a batch of n=8 per group and see if microdialysis measurements are sufficient and if we can measure sufficient neurons (minimum n=18) during electrophysiology. Simultaneously we will conduct pilot studies of magnetothermal DBS in rats in the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA) (not in this protocol).

Refinement
To learn and refine the procedures of microdialysis and electrophysiology we will firstly conduct these experiments at the department of neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.). This will reduce the amount of errors. The current DBS technique is widely used in our lab so this technique is already refined. We will use general anesthesia during insertion of current DBS electrodes together with the insertion of a single-cannula for microdialysis and for electrophysiological recordings in their last experiment. All animals will receive appropriate analgesics during post-operative recovery when needed. Too further refine our experiment we will insert different human endpoints.

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

All animals will be under general anaesthesia during insertion of the current DBS electrodes, implementation of the cannula for microdialysis and for electrophysiological recordings. Part of the animals will be sacrificed after microdialysis using an overdose of pentobarbital and consecutive transcardial perfusion. Electrophysiological recordings will be done during their last experiment and all animals will undergo an overdose of pentobarbital followed by consecutive transcardial perfusion. To minimalize suffering we will insert human endpoints in our protocol as described below.

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.

It is just recently discovered that current DBS of the vmPFC causes antidepressant effects in an experimental depression model in rats [7]. Since we will subdivide the vmPFC into the infralimbic, prelimbic and dorsal penduncular cortex we will be the first to further investigate stimulation of these different parts together with monoamine concentration changes and electrophysiological changes in the microcircuit involved in this stimulation paradigm. Although microdialysis and electrophysiological recordings in an experimental depression model for rats has been performed before, investigating the involved microcircuits of the prefrontal cortex is a new approach [14].

Microdialysis and electrophysiological recordings will firtsly be trained at the department of

neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.). [11]. This will reduce the amount of errors.

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

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

All animals will be housed individually.

For the chronic unpredictable stress (CUS) model all animals will undergo variable stressors for the maximal duration of ten weeks. We will vary the stressors daily to induce chronic stress, which is the basis of our research. For this animal model indivual housing is nescesarry since it enhances susceptiblility to these chronic stressors [13].

We will integrate the insertion of DBS electrodes and a microdialysis cannula in this animal model of experimental depression.

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?

X ☐ 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.

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

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

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

General anesthesia will be used during the stereotaxic insertion of the DBS electrodes and the cannula insertion for microdialysis later on. Animals will receive appropriate analgesics during their post-operative recovery period. All animals will be monitored daily during the experimental procedures and will be checked for signs of pain or too much discomfort. Taking into consideration that here will be severe discomfort using the CUS animal model. In case animals show pain during any of the experimental procedures described, they will receive analgesics. All animals will get general anesthesia during euthanasia after microdialysis or during electrophysiological measurement.

To further minimalize pain or suffering we will insert human endpoints in our protocol as described below.

I. Other aspects compromising the welfare of the animals

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

Animals will experience chronic stress by adding variable stressors during our experimental model of depression. Our research is based on experimental depression and the previous mentioned validated animal model for experimental depression includes exposure to chronic stress.

Animals will experience mild additional stress during microdialysis.

Another adverse effect of stereotactic insertion of the current DBS electrodes and the cannula for microdialysis could be loss of an electrode or cannula construct.

Explain why these effects may emerge.

Stress during our experimental depression animal models will occur since we will apply daily stressors such as; tilt cages, soiled bedding or stroboscopic light. Additional stress will emerge when executing CSF sampling during microdialysis.

Loss of an electrode or cannula construct might be due to scratching.

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

During our CUS and CSDS model, all animals will be inspected daily. When an animal shows too much stress, i.e. a disrupted physical appearance together with disrupted unprovoked behaviour and or abnormal responses to external stimuli and or self-induced trauma, we will remove it from the experiment and euthanize it consecutively. This will be discussed in more detail in our humane endpoints and working protocol.

To prevent loss of the electrodes or microdialysis cannula, we will secure them to the skull in the right position using dental cement. We will give all animals a post-operative recovery-period that allows the skin around the electrodes and cannula to recover. During our recovery period and experiment, all animals will be inspected daily.

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.

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

Human endpoints will mainly be based on general considerations regarding general health, too much discomfort in the CUS animal model or complications of stereotactic surgery.

Human endpoints will include:

-Signs of a disturbed physical appearance, disturbed unprovoked behavior, self-induced trauma or moderately abnormal responses to external stimuli.

-Rapid or progressive weight loss together with a loss of body condition compared to the beginning of our experiments. During our experiment we will inspect all animals daily using the general impression and physiological condition score format. We will weight all animals several times a week. Only measuring weight changes or disturbances in body condition is not a good way to

interpret general health and therefore we will use both measurements scales together[5].

-Signs of untreatable local or generalized infection for more than 3 days.

If a human endpoint is reached we will take the animal out of the experiment, euthanize it with an overdose of pentobarbital follwed by transcardial perfusion for post-mortem analysis. At the end of the experiments, all animals will be sacrificed by an overdose of pentobarbital follwed by transcardial perfusion for post-mortem brain analysis.

Indicate the likely incidence.

We estimate the likely incidence at maximally 25%, for this reason we included this percentage of dropouts in our maximal estimated number of animals.

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').

All animals will undergo moderate discomfort following surgery.
75% will undergo severe discomfort during the induced depression and following experiments while 25% of all animals will undergo mild discomfort as they serve as non-depressed controls.

Surgery:
The level of discomfort following stereotactic insertion of current DBS electrodes will be moderate and the insertion of the cannula for microdialysis will be moderate as well.

CUS animal model:
The expected level of discomfort due to the CUS animal model used and individual housing is severe. This animal model of experimental depression will give a high level of stress during several consecutive weeks.

Deep brain stimulation:
The level of discomfort following DBS will be mild.

Microdialysis:
The level of discomfort during microdialysis will be mild. We will collect samples of CSF before, during and after stimulation in free moving animals using the implanted cannula.

Euthanasia:
The level of discomfort during euthanasia by an overdose of pentobarbital followed by transcardial perfusion will be mild. Euthanasia will follow after microdialysis.

Electrophysiology:
The level of discomfort during electrophysiological measurements will be mild since all animals will be under general anaesthesia during this procedure. This is followed by an overdose of pentobarbital and consecutive transcardial perfusion.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

All animals will be sacrificed at the end of the experiments right after electrophysiological

measurements. We will isolate the brains to check for the correct electrode, cannula and recording probe localisation and to perform immunohistochemistry in a later stage if needed.

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.

x☐ Yes

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11. Tan, S.K., H. Hartung, V. Visser-Vandewalle, H.W. Steinbusch, Y. Temel, and T. Sharp, *A combined in vivo neurochemical and electrophysiological analysis of the effect of high-frequency stimulation of the subthalamic nucleus on 5-HT transmission.* Exp Neurol, 2012. **233**(1): p. 145-53.
12. van Goethem, N.P., K. Rutten, F.J. van der Staay, L.A. Jans, S. Akkerman, H.W. Steinbusch, etal.J. Prickaerts, *Object recognition testing: rodent species, strains, housing conditions, and estrous cycle.* Behav Brain Res, 2012. **232**(2): p. 323-34.
13. Willner, P., *Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS.* Neuropsychobiology, 2005. **52**(2): p. 90-110.
14. Yang, L.M., B. Hu, Y.H. Xia, B.L. Zhang, and H. Zhao, *Lateral habenula lesions improve the behavioral response in depressed rats via increasing the serotonin level in dorsal raphe nucleus.* Behav Brain Res, 2008. **188**(1): p. 84-90.

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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
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: Stimulation of the different regions of the vmPFC using magnetothermal DBS

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 experimental approach of this study is to apply magnetothermal deep brain stimulation (DBS) in the different parts of the ventromedial prefrontal cortex (vmPFC) in rats undergoing the chronic unpredictable stress (CUS) model and to identify which specific part of the vmPFC causes which specific antidepressant effects [4, 6, 7]

Firstly we will sensitize neurons to heat in either the infralimbic, prelimbic or dorsal peduncular cortex using a stereotactic injection of a lentivirus. This procedure takes place under general anesthesia. Animals will be given a post-operative recovery period.

Secondly, to gain depressive-like behaviour in our animals we will use the chronic unpredictable stress (CUS) model. For the CUS model, all animals will be exposed to a certain period of stress.

After a period of stress we will inject nanoparticles or a nanoparticle free solution under general anaesthesia using the same coordinates as we did for injection of the lentivirus. We will inject in the same region as the lentivirus so either inject in the infralimbic, prelimbic or dorsal peduncular cortex. Sham control animals will be injected with a nanoparticle free solution.

After a post-operative recovery period we will start magnetothermal DBS by exposure to an alternating magnetic field. This alternating magnetic field causes a fast vibration of the magnetic nanoparticles. These fast

movements generate heat and activate the heat-sensitive capsaicin receptors. Activation of these receptors causes neural excitation due to the influx of ions. The stimulation parameters for this study will be derived from results gained at the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). All animals will undergo behavioural testing when exposed to the magnetic field. We will also add control groups not being exposed to a magnetic field. We will test antidepressant effects of magnetothermal DBS of subregions in the vmPFC by comparing behavioural outcomes between experimental and control groups. The maximal duration of stress including behavioural testing will be ten weeks.

Consecutively we want to identify the neural microcircuits influenced by magnetothermal DBS. Based on our previous experiments, we will be able to focus on particular potent brain regions.

We will visualize these specific brain regions and recent activated neurons by using in vivo rodent PET-CT approaches, post-mortem functional anatomical mapping, immediate early gene mapping and immunohistochemical staining's as is done before for current DBS. After PET-CT we will perfuse all animals and fixate their brains for post-mortem analysis. PET-CT alone will not be enough to distinct specific brain regions since the spatial resolution is low and it is not possible to specify different types of cells. For this reason, also stated in appendix 1, animals cannot function as their own control and a different control group is needed.

The primary outcome:

- Mood- and movement related behavioural parameters tested with a battery of behavioural tasks. We will compare outcomes between the experimental and control groups.

Secondary outcomes:

- PET-CT images and their alterations between experimental and control groups.
- Neuronal alterations between experimental and control groups using post mortem immunohistochemical analysis. We will use various staining techniques (i.e. immediate early gene mapping, functional anatomical mapping, cell type and neurotransmitter specification) and quantify:
  - i) Changes in recent neuronal activity
  - ii) Changes in the number of neurons expressing different neurotransmitters
  - iii) Changes in the distribution of GABAergic, glutamatergic and dopaminergic markers.

We will scan multiple potent brain regions possibly involved in a microcircuit following DBS of the vmPFC.

With this information we will be able to compare results of this new magnethothermal DBS technique to the technique of current DBS. Magnetothermal DBS is a promising new therapy of neuromodulation since there is no need to insert electrodes and the area of activation and its parameters can be controlled more precisely. Its sensitivity and specificity is higher than that of current DBS [2]. Therefor we hypothesize that magnetothermal DBS is a better technique to unravel distinct microcircuitries responsible for different aspects of mood and mood-related behavior.

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

Surgery

During surgery we will inject lentivirus in either the infralimbic, prelimbic of dorsal peduncular cortex under general anesthesia using a stereotactic frame. With this lentivirus we sensitize neurons to heat by adding heat-sensitive receptors (TRPV) in the region of the injection. Animals will be given a post-operative recovery period.

Animal models

For our experiments we will use the CUS animal model. This model has been proven effective in mice and rats and is a well-validated animal models for experimental stress and depression [4, 6]. For the CUS model we will vary multiple unpredictable stressors during 3 consecutive weeks. These stressors will include a shift in hours of light per day, exposure to stroboscopic light (preferably 2.5 Hz), tilt cages, soiled bedding, paired housing and so on. During paired-housing, animals will be grouped in pairs with different partners. All stressors will last for 10-14 hours and since they should be unpredictable the exact

application times are not fixed. The stressors will always take place during the active period of the animal in a reversed day-night cycle in the laboratory. One will always take place in the morning while the other stressor will always take place in the afternoon or evening. We will continue these stressors during our DBS experiments and behavioural testing, but always apply these stressors after behavioural testing. The maximal duration of stressor exposure will be ten weeks.

Since magnetothermal DBS is now only working in anaesthetized mice, we need to extrapolate this method to awake rats. We are currently conducting pilot studies of magnetothermal DBS in awake rats (not in this protocol).

Injection of nanoparticles
After approximately three weeks of stress induced by our animal models we will inject nanoparticles or a nanoparticle free solution in the same region as the lentivirus under general anaesthesia. Nanoparticles will be obtained from a general supplier and will be injected using a stereotactic frame.

Magnetothermal DBS
After a post-operative recovery period, all animals will undergo magnetothermal DBS by exposure to a alternating magnetic field. In this method, specific neurons are sensitized to heat by lentiviral delivery of heat-sensitive receptors (TRPV). These receptors can be activated by magnetic nanoparticles using an alternating magnetic field. This alternating magnetic field causes a fast vibration of the magnetic nanoparticles generating heat in the near surrounded area. This heat activates the heat-sensitive capsaicin receptors causing neural excitation due to the influx of ions. This makes magnetothermal DBS a perfect method to remotely and wirelessly drive neural excitation. Parameters of the stimulation paradigm will be obtained during our exchange and pilot studies at MIT.

Behavioural testing
We will evaluate the effects of magnetothermal DBS of different parts the vmPFC by measuring different behavioral parameters linked to mood and cognition. It is interesting to gain further insight into the contribution of each specific region (infralimbic cortex, prelimbic cortex, and dorsal peduncular cortex) of the vmPFC and the underlying microcircuits involved.

Depression covers multiple modalities including anxiety, behavioral despair, lack in motivation, hedonia and changes in cognition [6, 11, 3, 8]. Traits of the disease vary from patient to patient. We want to discover if stimulation of different regions of the vmPFC changes different behavioral outcomes in different modalities. In this way we can personalize the stimulation paradigm in humans in the future based on their behavioral traits and the underlying mechanism responsible for these traits. For this reason, we will stimulate all three regions of the vmPFC.

All animals will undergo behavioural testing when exposed to the magnetic field with or without the addition of nanoparticles. A summary of the different behavioral tests and their readout parameters is listed below. Cumulative discomfort caused by consequent behavioural testing will be minimized by a wash out period between the different tests. The selection of tests may change in the course of our study due to analyses of previous tests or due to logistic reasons.

Table 1. **Summary of the different behavioural tests, readout parameters and discomfort.**

Test paradigm	**Item(s)**	**Readout parameter(s)**	**Discomfort**
Forced swim task	mood	immobility duration, latency to immobility, swimming duration, climbing duration.	Moderate discomfort, animals will be in water
Sucrose intake/preference	mood	volume of sucrose intake corrected for animal weight.	Mild discomfort, results in increased anxiety
Food intake	mood	volume of food intake corrected for animal weight.	Moderate discomfort, animals will receive restricted feeding regimes or deprivation
Open field	anxiety	total distance travelled, average	Mild discomfort,

	and locomotion	speed and time spent in corners.	results in increased anxiety
Elevated zero maze	anxiety	time spent in closed arms	Mild discomfort, results in increased anxiety
Home-cage emergency	impulsivity and anxiety	latency to escape	Mild discomfort, results in increased anxiety
Barnes maze	memory	number of errors per trial, rate of decline in number of errors and path length.	Mild discomfort, results in increased anxiety
Morris water maze	memory	time spent in the platform quadrant, escape latency and swimming speed.	Moderate discomfort, animals will be in water
Cued and contextual fear conditioning	memory	percentage of time spent freezing and time to extinction.	Moderate discomfort, small electrical shock will be administered
Object location task	memory	ratio of time spent at object in new versus old location.	Mild discomfort, results in increased anxiety
Object recognition task	memory	ratio of time spent at new versus old object.	Mild discomfort, results in increased anxiety
Y-maze	memory	Ratio left-right discrimination	Mild discomfort, results in increased anxiety
Skinner box paradigms e.g. reaction time and attention tasks	cognition	reaction time, incorrect responses, correct responses, premature responses	Moderate discomfort, animals will receive restricted feeding regimes or deprivation

PET-CT and immunohistochemistry
All animals will be anaesthetized after behavioural testing to perform in vivo PET-CT and will get an overdose of pentobarbital followed by transcardial perfusion to isolate en preserved their brain for further post-mortem analysis. We will perform functional anatomical mapping, immediate early gene mapping (ie. C-fos) and immunohistochemical staining's in potent brain regions based on previous findings in our first experiments. With the results of our first experiments we can refine our search. The different techniques enable us to quantify:

i) Changes in recent neuronal activity after DBS.
ii) Changes in the number of neurons expressing different neurotransmitters after DBS.
iii) Changes in the distribution of GABAergic, glutamatergic and dopaminergic markers after DBS.

With this research we will be able to identify which brain regions are activated after magnetothermal DBS of different parts of the vmPFC. We will investigate if magnetothermal DBS also gives rise to antidepressant effects as is seen with current DBS and compare their activated microcircuits and behavioral outcomes to discover differences and similarities between these two stimulation paradigms

We will be able to investigate if magnetothermal DBS is indeed a valuable new technique of neuromodulation.

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 needed, we considered published studies and other previous studies by our group [6]. To minimize the number of animals used, we will perform a power analyses. In each

group we will perfuse all animals and fixate their brains for post-mortem analysis. This is needed to specify neuronal changes between experimental and control groups. For this reason animals cannot function as their own control and a different control group is needed.

We are currently conducting pilot studies of magnetothermal DBS in awake rats (not in this protocol). We will conduct these experiments in rats when magnetothermal DBS is successfully applied in rats at MIT.

B. The animals

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

Species

We will use adult male Sprague Dawley rats purchased from a registered breeding facility. We will use these animals since we have experience with them in our group and because they can be used in the CUS model.

Since magnetothermal DBS is now only working in mice, we need to extrapolate this method to rats. We are currently starting pilot studies of magnetothermal DBS in awake rats in the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA) (not in this protocol).

Gender

We will use only male rats since previous research has shown that the estrogen cycle of female rodents can interfere with behavior and the release of neurotransmitters [1, 9]. Their estrogen cycle will cause a larger spread in behavioral outcomes, which will interfere with our readout measurements [9].

Number of animals

The estimation of the number of animals needed per group is based on a pilot study of magnetothermal DBS done at MIT and from previous published research done in our research group using current DBS in animal models of depression [6, 2]. In their study of magnetothermal DBS they used anesthetized mice. As a proof of principle they showed that magnetothermal DBS is able to modulate predefined neurons in anesthetized mice. We will apply this method in awake rats undergoing behavioral testing. We will use a maximum of 15 animals per group based on both before mentioned studies.

Before starting experiments, we will perform a power analysis and take into account a possible drop out of animals. Animal dropout during our experiments can be due to: surgical complications, electrode loss, incorrect electrode localization, premature termination of the experiments due to too much stress during the CUS model, wrong injection of nanoparticles or errors during magnetic activation of these nanoparticles. We will take into account a dropout of 25% and aim to reduce this number by pilot studies during our exchange to MIT (not in this protocol).

Since magnetothermal DBS for now has only been tested in rats, we need to extrapolate this method to rats.

We will use 'depressed' animals with the CUS model and 'non-depressed' animals to function as their control. Each group will consist of a subgroup receiving magnetothermal DBS and a subgroup functioning as sham control. We will implant the electrodes in three different subregions of the vmPFC. We will use a maximal amount of 15 animals per group. This will lead to: the experimental group CUS+ with magnetothermal DBS (infralimbic=15, prelimbic=15, dorsal peduncular=15; n=45) and their sham controls (n=10). And the naïve group CUS- with magnetothermal DBS (n=45) and their sham controls (n=10). Taken together this will include 110 animals (Fig. 1). Furthermore, a possible 25% dropout needs to be taken into consideration. Taken all this into account we will use a maximal amount of 138 rats.

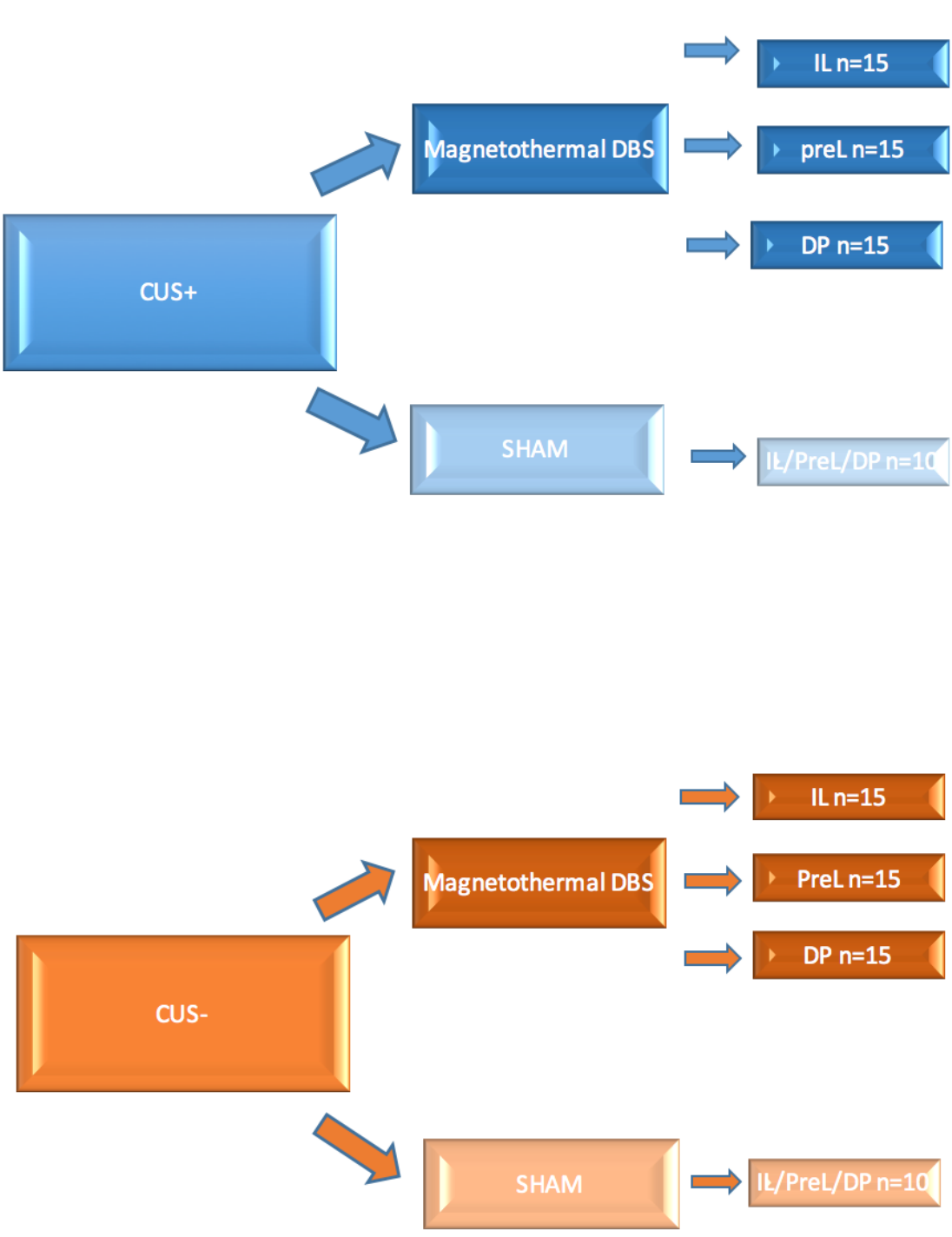

Fig. 1. Subdivision of groups for magnetothermal DBS.

C. Re-use

Will the animals be re-used?

x☐ 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.

Replacement
The primary aim of this study is to evaluate the influence of magntothermal DBS of different parts of the vmPFC on different behavioural parameters linked to mood. This cannot be achieved using in vitro experiments or in computer models since these models don't allow behavioral testing. Since the underlying microcircuits linked to this specific mood-related behavior are not known, we need the complex neural network of an animal model. This study cannot be performed in humans since the sensitization of neurons for now still needs lentiviral delivery of TRPV, which is not ethically approved in humans. In future research we will try to overcome the use of a lentivirus.

Reduction
We have chosen validated and well-known models of experimental stress and depression used before in our laboratory. In this way we limit the number of animals in our study since differences in specific behavioural-parameters following current DBS has been shown before. We hypothesize that magnetothermal DBS will also have these effects. We have limited the number of animals needed based on previous results of the influence of current DBS on behavioural parameters and on adequately working wireless magnetothermal DBS of the ventral tegmental area (VTA) [6, 2]. The intermediate results of our previous studies could further reduce group size if possible.

Refinement
To refine magnetothermal DBS we will first train the experimental procedure under supervision of the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). This new method makes it possible to selectively modulate specific populations of neurons and to control its parameters more precisely. This will help to gain a more sophisticated understanding as to which specific regions play a key role in network dysfunction in mood disorders. We will use general anesthesia for surgical procedures and PET-scan. We will use an overdose pentobarbital followed by consecutive transcardial perfusion. Both will minimize animal discomfort to a minimum. All animals will receive appropriate analgesics during their post-operative recovery period when needed.
As described before, we need to use the CUS model to gain depressive-like behavior. The intermediate results of our previous studies could refine this model. Too further refine our experiment we will insert different human endpoints.

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

All animals will be under general anaesthesia during stereotactic lentiviral delivery of heat-sensitive receptor TRPV and injection of magnetic nanoparticles into these brain regions later on. They will all get a post-operative recovery period. All animals will receive appropriate analgesics during their post-operative recovery period when needed. At the end of the experiment we will anaesthetize all animals before PET-CT followed by an overdose pentobarbital and consecutive transcardial perfusion. In this way discomfort is kept to a minimum. To minimize suffering in our animal models we will insert human endpoints in our protocol as described below.

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 procedure of magnetothermal DBS is very new and so far has only been preformed as a proof of principle at the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). It has not been used in an animal model for experimental depression before. By combining these two topics we will provide a new strategy bringing the research field of neurosurgery, advanced nanotechnology and psychiatry closer together. We might be able to develop a novel therapeutic strategy for patients with severe mood disorders and fine-tune neuromodulation using magnetothermal DBS.

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

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

All animals will be housed individually.

For the chronic unpredictable stress (CUS) model all animals will undergo variable stressors for the maximal duration of ten weeks. We will vary the stressors daily to induce chronic stress, which is the basis of our research. For this animal model indivual housing is nescesarry since it enhances susceptiblility to these chronic stressors [10].

We will integrate the insertion of microdialysis cannula in both animal models of experimental depression.

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?

X ☐ 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.

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

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

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

General anesthesia will be used during stereotactic lentiviral delivery of heat-sensitive receptor TRPV and injection of magnetic nanoparticles into these brain regions later on. All animals will receive appropriate analgesics during post-operative recovery period when needed. Animals will be monitored daily during the experimental procedures stated in section 2A and will be checked for signs of pain or discomfort taken into account that there will be discomfort using the CUS animal model. PET-CT will be performed under general anesthesia followed by an overdose of pentobarbital and consecutive transcardial perfusion. This will reduce animal discomfort to a minimum. To further minimalize suffering we will insert human endpoints in our protocol as described below.

I. Other aspects compromising the welfare of the animals

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

All animals will experience chronic stress by adding variable stressors during our experimental model of depression. Our research is based on experimental depression and the previous mentioned validated animal model for experimental depression includes exposure to chronic stress.

Animals might experience additional stress during the battery of behavioural tasks.

Furthermore, there could be a possible higher risk of infection during the time between the injection of the lentivirus and the insertion of nanoparticles in the same region four weeks later.

Explain why these effects may emerge.

Stress during our experimental depression animal models will occur since we will apply daily stressors such as; tilt cages, soiled bedding or stroboscopic light. Additional stress will emerge when executing particular behavioural tasks such as the forced swim task.

After lentiviral injection we need to keep an access to the same brain region to inject nanoparticles four weeks later.

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

During our CUS model, all animals will be inspected daily. When an animal shows too much stress, i.e. a disrupted physical appearance together with disrupted unprovoked behaviour and or abnormal responses to external stimuli and or self-induced trauma, we will remove it from the experiment. This will be discussed in more detail in our humane endpoints and working protocol.

Cumulative discomfort caused by consequent behavioural testing will be minimized by a wash out period between the different tests.

To prevent infection, we will cover up the injection side with a plastic cover slip and dental cement, which can be taken off during the injection of nanoparticles. Animals will be inspected daily during the post-operative recovery period and following experiments.

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.

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

Human endpoints will mainly be based on general considerations regarding general health, too much discomfort in the CUS animal model, complications of the injection of lentivirus into the brain or complications during stereotactic insertion of the nanoparticles.

Human endpoints will include:

-Signs of a disturbed physical appearance, disturbed unprovoked behavior, self-induced trauma or moderately abnormal responses to external stimuli.

-Rapid or progressive weight loss together with a loss of body condition compared to the beginning of our experiments. During our experiment we will inspect all animals daily using the general impression and physiological condition score format. We will weight all animals several times a week. Only measuring weight changes or disturbances in body condition is not a good way to interpret general health and therefore we will use both measurements scales together[5].

- Signs of untreatable local or generalized infection for more than 3 days.

- Major bleeding due to stereotactic lentiviral or nanoparticle injections.

If a human endpoint is reached we will take the animal out of the experiment, euthanize it with an overdose of pentobarbital follwed by transcardial perfusion for post-mortem analysis. At the end of the experiments, all animals will be sacrificed by an overdose of pentobarbital and consecutive transcardial perfusion for post-mortem brain analysis.

Indicate the likely incidence.

We estimate the likely incidence at maximally 25%, for this reason we included this percentage of dropouts in our maximal estimated number of animals.

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').

All animals will undergo moderate discomfort following surgery.
75% will undergo severe discomfort during the induced depression and following experiments while 25% of all animals will undergo mild discomfort as they serve as non-depressed controls.

Surgery:
The level of discomfort following stereotactic lentiviral TRPV delivery and stereotactic injection of nanoparticles later on will be moderate. Both procedures will take place under general anaesthesia.

CUS animal model:
The expected level of discomfort due to the CUS animal model and individual housing is severe. This animal model of experimental depression will give a high level of stress during several consecutive weeks.

Wireless magnetothermal deep brain stimulation:
The level of discomfort following magnetothermal DBS will be mild.

Behavioural testing:
The level of discomfort during our behavioural testing will be mild to moderate, see table 1. Cumulative discomfort caused by consequent behavioural testing will be minimized by a wash out period between the different tests.

Euthanasia:
At the end of our experiments all animals will undergo general anaesthesia, PET-CT, an overdose of pentobarbital followed by transcardial perfusion for brain preservation. This will give mild discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

All animals will be sacrificed at the end of the experiments to isolate their brain and perform functional anatomical mapping, immediate early gene mapping and immunohistochemical stainings.

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.

x☐ Yes

1. Barth, C., A. Villringer, and J. Sacher, *Sex hormones affect neurotransmitters and shape the adult female brain during hormonal transition periods.* Front Neurosci, 2015. **9**: p. 37.
2. Chen, R., G. Romero, M.G. Christiansen, A. Mohr, and P. Anikeeva, *Wireless magnetothermal deep brain stimulation.* Science, 2015. **347**(6229): p. 1477-80.
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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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University

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
4	Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression using magnetothermal DBS

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 experimental approach of this study is to measure changes in extracellular levels of the main monoamines and changes in electrophysiological properties in potent brain regions during magnetothermal DBS of the different parts of the vmPFC [9, 2]. In our previous experiment we aim to discover activated brain regions during our stress protocol and DBS of the vmPFC, therefore involved in the microcircuit of depressive-like behavior. Quantification of these potent brain regions will take place using post-mortem brain tissue of our previous experimental group (appendix 3). Only the brain regions mostly activated during our animal model of depression and furthermore accessible for electrophysiology or microdialysis will undergo these measurements during their experiment. We will include sham control groups not receiving actual stimulation and sham control groups not experiencing stress from the CUS animal model.

As described in appendix 3, we will firstly sensitize neurons to heat in either the infralimbic, prelimbic or dorsal peduncular cortex using stereotactic injection of a lentivirus. This procedure takes place under general anesthesia. Additionally, in part of the animals, we will insert a cannula in a brain region of interest that is involved in the microcircuits of depressive-like behaviour in our animal model. In each experimental group we will target a different brain region. Which regions are of interest is to be determined in our previous experiment. We will explore a maximum of three potent brain regions. If only one or two regions are of interest after our previous experiments including immunohistochemistry, we will only test these brain regions. The implanted cannula will serve as an access point for microdialysis. Animals will be given a post-operative recovery period.

Secondly, we will gain depressive-like behaviour in animals using the chronic unpredictable stress (CUS) model.

After a period of stress, determined in our first experiment, we will inject nanoparticles or a nanoparticle free solution under general anaesthesia using the same stereotactic coordinates as we did for injection of the lentivirus. We will inject in the same region as the lentivirus so either inject in the infralimbic, prelimbic or dorsal peduncular cortex. Sham control animals will be injected with a nanoparticle free solution.

After a post-operative recovery period we will start magnetothermal DBS by exposure to an alternating magnetic field. This alternating magnetic field causes a fast vibration of the magnetic nanoparticles. These fast movements generate heat and activate the heat-sensitive capsaicin receptors. Activation of these receptors causes neural excitation due to the influx of ions. All animals will undergo behavioural testing when exposed to the magnetic field. We will also add control groups not being exposed to a magnetic field. We will test antidepressant effects of magnetothermal DBS of subregions in the vmPFC by comparing behavioural outcomes between experimental and control groups. The maximal duration of stress including behavioural testing will be ten weeks.

In addition to magnetothermal DBS, microdialysis probes will be guided through the cannula and intracranial sampling of neurotransmitters will be performed. Measurements will take place before, during and after stimulation. The other part of the animals, not implanted with a cannula, will undergo electrophysiological recordings of different regions of interest under general anesthesia. So for all animals either microdialysis or electrophysiological measurements will be their final experiment followed by euthanasia with an overdose of pentobarbital and consecutive transcardial perfusion to preserve the brain for post-mortem analyses. We will measure shortly before, during and shortly after DBS. Which regions are of interest is to be determined in our previous experiment explained in appendix 1.

The primary outcomes:

- Changes in monoamine levels upon magnetothermal DBS of the different parts of the vmPFC.
- Changes in electrophysiological properties upon magnetothermal DBS of the different parts of the vmPFC.

We will investigate the most potent brain regions involved in the underlying microcircuit following magnetothermal DBS of the vmPFC observed in our previous experiment.

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

For this experimental design we will use experimental groups receiving the new technique of magnetothermal DBS in different regions of the vmPFC. Simultaneously we will measure the changes in monoamine concentrations levels and electrophysiological properties in different brain areas linked to stimulation of different parts of the vmPFC using microdialysis and electrophysiological recordings.

Surgery
We will inject lentivirus in either the infralimbic, prelimbic of dorsal peduncular cortex under general anesthesia using a stereotactic frame. With this lentivirus we sensitize neurons to heat by adding heat-sensitive receptors (TRPV) in the injected brain region. In a part of these animals, we will additionally insert a cannula in a brain region of interest for additional microdialysis measurements. A part of the animals will not be implanted with a cannula and will undergo electrophysiology instead of microdialysis. Animals will be given a post-operative recovery period.

Animal models
For our experiments we will use the CUS animal model. This model has been proven effective in mice and rats and is a well-validated animal models for experimental stress and depression [4, 7]. For the CUS model we will vary multiple unpredictable stressors during 3 consecutive weeks. These stressors will include a shift in hours of light per day, exposure to stroboscopic light (preferably 2.5 Hz), tilt cages,

soiled bedding, paired housing and so on. During paired-housing, animals will be grouped in pairs with different partners. All stressors will last for 10-14 hours and since they should be unpredictable the exact application times are not fixed. The stressors will always take place during the active period of the animal in a reversed day-night cycle in the laboratory. One will always take place in the morning while the other stressor will always take place in the afternoon or evening. We will continue these stressors during our DBS experiments. The maximal duration of stressor exposure will be ten weeks; including the days of experimental microdialysis or electrophysiology.

We are interested in the underlying mechanism of depression and its microcircuits; therefore the CUS models is the appropriate models to investigate our research questions.

Injection of nanoparticles
After the same duration of stress used in our previous experiments, we will inject nanoparticles or a nanoparticle free solution in the same region as we the injected lentivirus. This procedure is executed under general anaesthesia. Nanoparticles will be obtained from the same supplier stated in appendix 3. All animals will get a post-operative recovery period.

Magnetothermal DBS
After a post-operative recovery period, all animals will undergo magnetothermal DBS by exposure to a alternating magnetic field. In this method, specific neurons are sensitized to heat by lentiviral delivery of heat-sensitive receptors (TRPV). These receptors can be activated by magnetic nanoparticles using an alternating magnetic field. This alternating magnetic field causes a fast vibration of the magnetic nanoparticles generating heat in the near surrounded area. This heat activates the heat-sensitive capsaicin receptors causing neural excitation due to the influx of ions. This makes magnetothermal DBS a perfect method to remotely and wirelessly drive neural excitation. Parameters of the stimulation paradigm will be obtained during our exchange and pilot studies at MIT and our previous experiments described in appendix 3.

Microdialysis and electrophysiology
In addition to magnetothermal DBS we will conduct microdialysis or electrophysiological recordings of different parts of the microcircuits discovered in our previous experiments. We will collect CSF samples via the cannula implanted during stereotactic surgery. In these samples we will analyse different monoamine concentrations to discover what changes during magnetothermal DBS of different parts of the vmPFC. Part of the animals will be sacrificed after microdialysis using an overdose of pentobarbital followed by transcardial perfusion. The other part of animals will undergo general anaesthesia for electrophysiological recordings of other potent brain regions found in our previous experiments. Since the animals are under general anaesthesia scanning multiple brain regions will not increase the level of discomfort. The amount of animals per group is to be determined in our previous experiments. This is their final experiment so after this experiment all animals will be sacrificed using an overdose of pentobarbital followed by transcardial perfusion.

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 needed, we considered the published study of magnetothermal DBS in mice and previous research done in our laboratory using current DBS in the vmPFC of rats and research performing microdialysis and electrophysiology of different brain regions in rats [6, 7, 9, 2].

B. The animals

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

Species

We will use adult male Sprague Dawley rats purchased from a registered breeding facility. We will use these animals since we have experience with them in our group, because they can be used in the CUS model and because microdialysis and electrophysiology can be performed on these animals [7, 9]. Beforehand, we will conduct pilot studies of magnetothermal DBS in awake rats in the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA) (not in this protocol). Rats provide much more reliable data compared to mice [3].

Gender

We will use only male rats since previous research has shown that the estrogen cycle of female rodents can interfere with behavior and the release of neurotransmitters [1, 10]. Their estrogen cycle will cause a larger spread in behavioral outcomes, which will interfere with our readout measurements [10].

Number of animals

The estimation of the number of animals needed per group is based on a pilot study of magnetothermal DBS done at MIT together with previous published research concerning the CUS model and microdialysis in parallel with electrophysiological measurement [8, 7, 9]. We will use a maximum of 15 animals per group. We will have three different stimulation regions (infralimbic cortex, prelimbic cortex and dorsal peduncular cortex).

Before starting experiments, we will perform a power analysis and take into account a possible drop out of animals observed in our previous experiments. Animal dropout during our experiments can be due to: surgical complications during injection of the lentivirus or injection of the nanoparticles, cannula loss, too much stress during the CUS model or complications during electrophysiology.

Since magnetothermal DBS for now has only been tested in mice, we need to extrapolate this method to rats. We are currently conducting pilot studies of magnetothermal DBS in rats.

This will lead to:

Microdialysis: the experimental group CUS+ with magnetothermal DBS (infralimbic, prelimbic, dorsal peduncular; n=45) in three potent brain regions (n=135), and their sham controls (n=30). And the naïve group CUS- with magnetothermal DBS (n=135) and their sham controls (n=30).

We will start with an animal batch of n=8 per group for microdialysis. If this group size already gives significant differences, we will leave the group size at n=8 in total and do no more microdialysis experiments. Also experience from our previous current DBS group will show us if n=8 animals per group is enough. The sham control group will then be n=5. This will reduce the amount of animals to n=174 for microdialysis.

Electrophysiology: the experimental group CUS+ with magnetothermal DBS (infralimbic, prelimbic, dorsal peduncular; n=45) in three potent brain regions (n=135), and their sham controls (n=30). And the naïve group CUS- with magnetothermal DBS (n=135) and their sham controls (n=30).

We will start with an animal batch of n=8 per group for electrophysiology. We need a particular amount of neurons for electrophysiology to measure electrophysiological differences upon stimulation between the different groups. Previous research has shown electrophysiological effects in the dorsal raphe nucleus upon vmPFC stimulation, using n=18 neurons [7]. We therefore will use a minimum of n=18 neurons. If eight animals per group will give enough neurons for statistical analysis, we will only test eight animals per group. Also previous experience will show us how many animals are needed for the desired number of neurons (appendix2). The sham control group will then be n=5. This will reduce the amount of animals to n=174 for electrophysiology.

Furthermore a possible 25% dropout needs to be taken into consideration.

Taken all this into account we will use n=435 rats, when n=8 per experimental group and n=5 per sham group already give measurable outcomes for microdialysis and enough neurons for electrophysiological measurements. If this is not the case, we will introduce another animal batch per group resulting in the maximum amount of 825 rats.

Further reduction may follow in our working protocol after previous experiments has shown us the most potent regions of interest to measure chemical and electrophysiological changes.

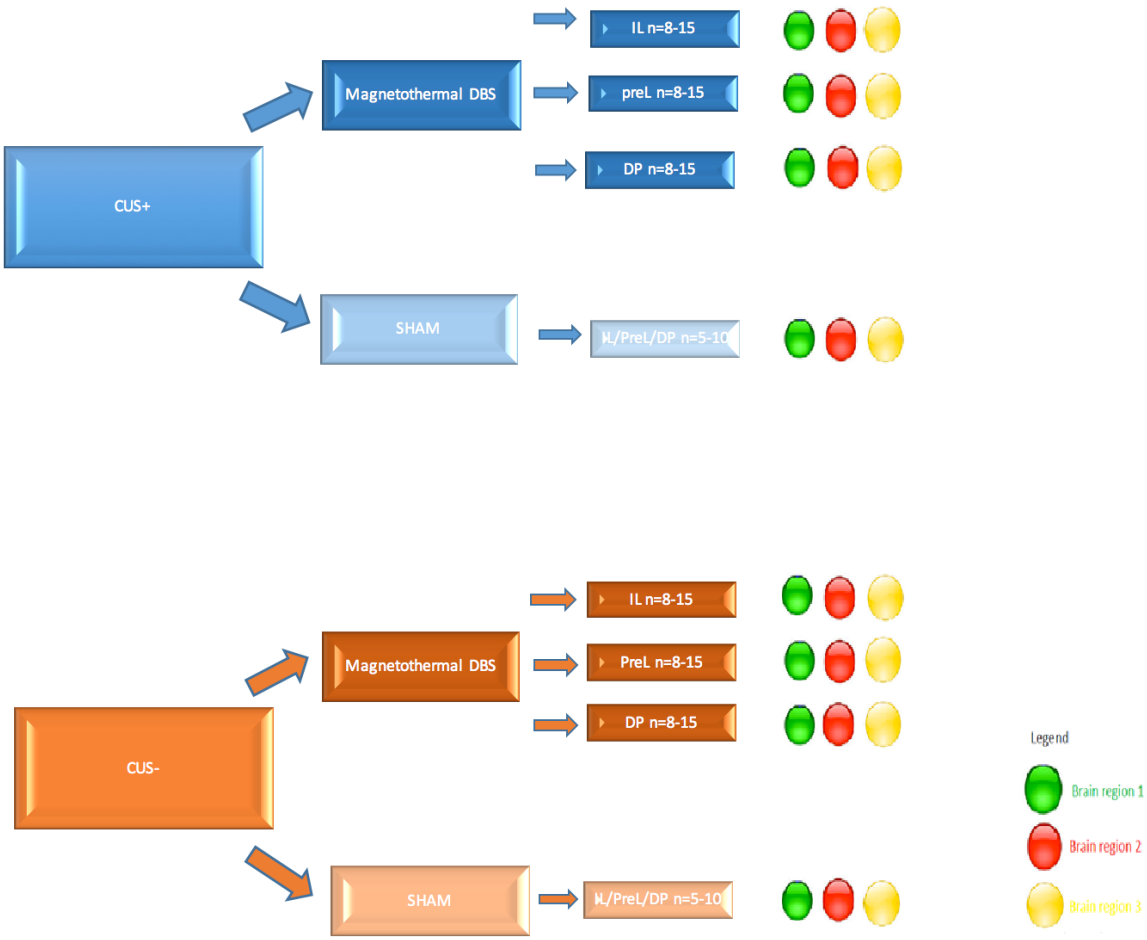

Fig. 1. Subdivision of groups for Magnetothermal DBS and additional microdialysis (n=8-15) or electrophysiology (n=8-15), together with a sham control group (n=5-10). Each brain region indicated with a green, red or yellow circle, will either be a microdialysis experiment or an electrophysiological experiment.

C. Re-use

Will the animals be re-used?

x☐ 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.

Replacement

The primary aim of this study is to investigate the effect of infralimbic cortex, prelimbic cortex and dorsal peduncular cortex magnetothermal DBS on the levels of monoamines and electrophysiological properties in different forebrain regions. This will help us to uncover the underlying mechanisms of antidepressant effects of the new technique of magnetothermal DBS in the vmPFC. This cannot be achieved using in vitro experiments or in computer models since we need a complex 'depressed' neural microcircuit to detect changes in monoamine levels and electrophysiological properties. This study cannot be performed in humans since this procedure with a lentivirus at this stage of research will not be ethically approved.

Reduction
We have chosen a validated and well-known model of experimental stress and depression used before in our laboratory. In this way we limit the number of animals in our study since differences in specific behavioural-parameters following current DBS has been shown before. We hypothesize that magnetothermal DBS will also have these effects and that underlying changes in monoamines and electrophysiological properties can therefore also be detected. We have limited the number of animals based on previous results of magnetothermal DBS in mice, current DBS in the vmPFC of rats and microdialysis together with electrophysiological measurements in different brain regions [6, 8, 9, 2]. To further reduce the amount of animals we will start with a batch of n=8 per group and see if microdialysis measurements are sufficient and if we can measure sufficient neurons (minimum n=18) during electrophysiology. The intermediate results of our previous studies could further reduce group size if possible. If previous experiments show that only particular forebrain regions are of interest to measure monoamine levels and electrophysiological properties, we will only measure these specific brain regions.

Refinement
To refine magnetothermal DBS we will first train the experimental procedure under supervision of the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). To refine the procedures of microdialysis and electrophysiology we will firstly conduct these at the department of neuropharmacology at the university of Oxford (Univ. of Oxford, Oxford, U.K.). Both will reduce the amount of errors.
We will use general anesthesia during the insertion of the lentivirus, the insertion of nanoparticles, the insertion of the cannula for microdialysis and during electrophysiology. All animals will receive appropriate analgesics during post-operative recovery when needed. Too further refine our experiment we will insert different human endpoints.

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

All animals will be under general anaesthesia during; stereotactic injection of the lentivirus, stereotactic injection of magnetic nanoparticles into these brain regions later on, implementation of the cannula for microdialysis and during electrophysiological recordings. They will all get a post-operative recovery period. All animals will receive appropriate analgesics during their post-operative recovery period when needed. All animals will be euthanized with an overdose of pentobarbital before transcardial perfusion, either after microdialysis of electrophysiological recordings. To further minimize suffering in our animal models we will insert human endpoints in our protocol as described below.

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.

As stated and motivated in appendix 3, the procedure of magnetothermal DBS is very new and so far has only been preformed as a proof of principle at the research group of Materials Science and Engineering at the Massachusetts Institute of Technology (MIT, Boston, USA). It has not been used in an animal model for experimental depression. It would be the first experiment in which we will apply this new technique

of DBS and look at the involved microcircuits in mood related behavior. By combining a new nanotechnique of DBS with experimental depression will provide a new strategy bringing the research field of neurosurgery, advanced nanotechnology and psychiatry closer together. We might be able to develop a novel therapeutic strategy for patients with severe mood disorders and fine-tune neuromodulation using magnetothermal DBS.

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

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

All animals will be housed individually.

For the chronic unpredictable stress (CUS) model all animals will undergo variable stressors for the maximal duration of ten weeks. We will vary the stressors daily to induce chronic stress, which is the basis of our research. For this animal model indivual housing is nescesarry since it enhances susceptiblility to these chronic stressors [11].

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?

X ☐ 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.

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

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

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

General anesthesia will be used during stereotactic lentiviral delivery of heat sensitive receptor TRPV, stereotactic injection of magnetic nanoparticles into these brain regions, stereotactic implantations of a cannula for microdialysis and electrophysiological recordings.

Animals will receive appropriate analgesics during a post-operative recovery period. All animals will be monitored daily during the experimental procedures and will be checked for signs of pain or too much discomfort. Taking into consideration that here will be some discomfort using the CUS animal model. In case animals show pain during any of the experimental procedures described, they will receive

analgesics.

All animals will get general anesthesia during euthanasia or during electrophysiological measurements. Euthanasia will take place after microdialysis.

To further minimalize suffering we will insert human endpoints in our protocol as described below.

I. Other aspects compromising the welfare of the animals

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

All animals will experience chronic stress by adding variable stressors during our experimental model of depression. Our research is based on experimental depression and the previous mentioned validated animal model for experimental depression includes exposure to chronic stress.

Animals will experience mild additional stress during microdialysis.

Another adverse effect of stereotactic insertion of the cannula for microdialysis could be loss of a cannula construct.

Furthermore, there could be a possible higher risk of infection during the time between the injection of the lentivirus and the insertion of nanoparticles in the same region four weeks later.

Explain why these effects may emerge.

Stress during our experimental depression animal model will occur since we will apply daily stressors such as; tilt cages, soiled bedding or stroboscopic light. Additional stress will emerge when executing CSF sampling during microdialysis.

Loss of an electrode or cannula construct might be due to scratching.

After lentiviral injection we need to keep an access to the same brain region to inject nanoparticles four weeks later.

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

During our CUS model, all animals will be inspected daily. When an animal shows too much stress, i.e. a disrupted physical appearance together with disrupted unprovoked behaviour and or abnormal responses to external stimuli and or self-induced trauma, we will remove it from the experiment and euthanize it consecutively. This will be discussed in more detail in our humane endpoints and working protocol.

To prevent loss of a microdialysis cannula construct, we will secure them to the skull in the right position using dental cement. We will give all animals post-operative recovery-period that allows the skin around the cannula to recover. During our recovery period and experiment, all animals will be inspected daily.

To prevent infection, we will cover up the injection side with a plastic cover slip and dental cement, which can be taken off during the injection of nanoparticles. Animals will be inspected daily during the post-operative recovery period and following experiments.

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.

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

Human endpoints will mainly be based on general considerations regarding general health, too much

discomfort in the CUS animal model, complications of the injection of lentivirus into the brain or complications during stereotactic insertion of the nanoparticles.

Human endpoints will include:

-Signs of a disturbed physical appearance, disturbed unprovoked behavior, self-induced trauma or moderately abnormal responses to external stimuli.

-Rapid or progressive weight loss together with a loss of body condition compared to the beginning of our experiments. During our experiment we will inspect all animals daily using the general impression and physiological condition score format. We will weight all animals several times a week. Only measuring weight changes or disturbances in body condition is not a good way to interpret general health and therefore we will use both measurements scales together[5].

-Signs of untreatable local or generalized infection for more than 3 days: diarrhea, coughing and progressive weight loss.

- Major bleeding due to stereotactic lentiviral or nanoparticle injections.

If a human endpoint is reached we will take the animal out of the experiment, euthanize it with an overdose of pentobarbital, follwed by trasncardial perfusion for post-mortem analysis. At the end of the experiments, all animals will be sacrificed with an overdose of pentobarbital follwed by transcardial perfusion for post-mortem brain analysis.

Indicate the likely incidence.

We estimate the likely incidence at maximally 25%, for this reason we included this percentage of dropouts in our maximal estimated number of animals.

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').

All animals will undergo moderate discomfort following surgery.
75% will undergo severe discomfort during the induced depression and following experiments while 25% of all animals will undergo mild discomfort as they serve as non-depressed controls.

Surgery
The level of discomfort following stereotactic lentiviral TRPV delivery and stereotactic injection of nanoparticles four weeks later will be moderate. The insertion of the cannula for microdialysis will be moderate as well. All procedures will take place under general anaesthesia.

CUS animal model
The expected level of discomfort due to the CUS animal model used and individual housing is severe. This animal model of experimental depression will give a high level of stress during several consecutive weeks.

Wireless magnetothermal Deep brain stimulation
The level of discomfort following magnetothermal DBS will be mild.

Microdialysis:
The level of discomfort during microdialysis will be mild. We will collect samples of CSF before, during and after stimulation in free moving animals using the implanted cannula.

Euthanasia:
The level of discomfort during euthanasia with an overdose of pentobarbital followed by transcardial perfusion will be mild. Euthanasia will follow after microdialysis when electrophysiological measurements are no longer needed.

Electrophysiology:
The level of discomfort during electrophysiological measurements will be mild since all animals will be under general anaesthesia during this procedure. This procedure is followed by an overdose of pentobarbital and consecutive transcardial perfusion.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

All animals will be sacrificed at the end of the experiments right after electrophysiological measurements. We will isolate the brains to check for the lentiviral TRPV and nanoparticle localization, cannula and recording probe localisation and to perform immunohistochemistry in a later stage if needed.

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.

x☐ Yes

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AVD107002016542

Bijlagen
1

Datum 27 juni 2016
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 10 mei 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Magnetothermal and current deep brain stimulation in experimental depression" met aanvraagnummer AVD107002016542. Wij hebben uw aanvraag beoordeeld.

Op 7 juni en 28 juni 2016 heeft u uw aanvraag aangevuld. U heeft op de vragen van de CCD gereageerd. U heeft het aantal dieren in bijlage dierproeven en in de Niet technische samenvatting aangepast, de go/no-go momenten beschreven en een nieuwe versie van uw Niet-technische samenvatting naar de CCD toe gestuurd.

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. De voorwaarde betreffende het afstemmen van de go/no-go momenten met de IvD is gesteld in het kader van de 3V's, om te voorkomen dat dat dieren onnodig worden gebruikt in het geval dat de experimenten niet de verwachte resultaten opleveren. De algemene voorwaarde betreffende artikel 10, lid 1a van de wet wordt gesteld bij vergunningen met een langere looptijd. Dit om te voldoen aan datgene wat volgt uit dit artikel. U kunt met uw project "Magnetothermal and current deep brain stimulation in experimental depression" starten. De vergunning wordt afgegeven van 30 juni 2016 tot en met 1 juni 2021.

Overige wettelijke bepalingen blijven van kracht.

Beoordeling achteraf
Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1, lid 1d en lid 3, in de wet. Meer informatie over de eisen bij een beoordeling achteraf vindt u in de bijlage.
In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-UM gevoegd. Dit advies is opgesteld op 10 mei 2016. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Wij hebben de DEC om aanvullende informatie gevraagd. Op 30 mei 2016 heeft de DEC gereageerd op onze vragen. De DEC heeft het antwoord van de aanvrager op de gestelde vragen naar de CCD gestuurd.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie, nemen wij over, inclusief de daaraan ten grondslag liggende motivering. In aanvulling op het advies worden twee algemene voorwaarden gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving 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.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze:

[redacted]
Algemeen Secretaris

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

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Universiteit Maastricht
Adres:	Postbus 616
Postcode en plaats:	6200 MD MAASTRICHT
Deelnemersnummer:	10700

deze projectvergunning voor het tijdvak 30 juni 2016 tot en met 1 juni 2021, voor het project "Magnetothermal and current deep brain stimulation in experimental depression" met aanvraagnummer AVD107002016542, volgens advies van Dierexperimentencommissie DEC-UM. De functie van de verantwoordelijk onderzoeker is Professor neurochirurgie. Voor de uitvoering van het project is Onderzoeker verantwoordelijk.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 10 mei 2016
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 10 juni 2016;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 27 juni 2016;
c Advies van dierexperimentencommissie d.d. 10 mei 2016, ontvangen op 10 mei 2016.
d De aanvullingen op uw aanvraag, ontvangen op 7 juni en 28 juni 2016

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Stimulation of the different regions of the ventromedial prefrontal cortex (vmPFC) using current DBS	Ratten (Rattus norvegicus) / Spague Dawley	138	75,00% Ernstig 25,00% 25,00% Licht	'All animals will be housed individually. For the chronic unpredictable stress (CUS) model all animals will undergo variable stressors for the maximal duration of ten weeks. We will vary the stressors daily to induce chronic stress, which is the basis of our research. For this animal model indivual housing is nescesarry since it enhances susceptiblility to these chronic stressors [11].'
3.4.4.2 Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression using current DBS	Ratten (Rattus norvegicus) / Sprague Dawley	825	75,00% Ernstig 25,00% Licht	'All animals will be housed individually.'
3.4.4.3 lst·i~ulation of the different regions of the vmPFC using magnetothermal DBS	Ratten (Rattus norvegicus) / Sprague Dawley	138	75,00% Ernstig 25,00% Licht	'All animals will be housed individually.'
3.4.4.4 Microdialysis and electrophysiological recordings in the microcircuits of the vmPFC in experimental depression using magnetothermal DBS	Ratten (Rattus norvegicus) / Sprague Dawley	825	75,00% Ernstig 25,00% Licht	'All animals will be housed individually.'

Voorwaarden

Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen

In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.
Deze beoordeling zal uiterlijk juni 2022 plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst de dierproeven conform de vergunning waren.
De vergunning wordt verleend onder de voorwaarde dat 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.

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 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 13d 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 13c 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.
De Minister heeft vrijstelling ontheffing verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.

Beoordeling achteraf
Volgens artikel 10a1, lid 1d en lid 3 van de wet worden projecten waarbij niet-menselijke primaten worden gebruikt, projecten die als ernstig ingedeelde dierproeven omvatten of een dierproef die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, achteraf beoordeeld worden.

	Inventaris Wob-verzoek W16-21S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2016543								
1	Aanvraagformulier				x		x		
2	Projectvoorstel			x					
3	Niet-technische samenvatting oud			x					
4	Bijlage beschrijving dierproeven 1			x					
5	Bijlage beschrijving dierproeven 1			x					
6	DEC-advies				x		x		
7	Ontvangstbevestiging				x		x		
8	Verzoek aanvulling aanvraag				x		x		
9	Niet technische samenvatting herzien	x		x					
10	Adviesnota CCD		x						x
11	Beschikking en vergunning				x		x		

Centrale Commissie Dierproeven

1.

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* 10700
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	Universiteit Maastricht
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	50169181

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	Minderbroedersberg	4-6
Postbus	616	
Postcode en plaats	6200 MD	Maastricht
IBAN	NL04 INGB 0679 5101 68	
Tenaamstelling van het rekeningnummer	Universiteit Maastricht	

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☒ Dhr. ☐ Mw.
Functie	[redacted]	
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	[redacted] ☒ Dhr. ☐ Mw.
		Functie	[redacted]
		Afdeling	[redacted]
		Telefoonnummer	[redacted]
		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	1 - 3 - 2016
		Einddatum	1 - 3 - 2021
3.2	Wat is de titel van het project?	Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease	
3.3	Wat is de titel van de niet-technische samenvatting?	Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC UM
		Postadres	Postbus 616, 6200MD Maastricht
		E-mailadres	[redacted]

[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1441,00 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

☒

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 1.7). 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	
Plaats	Maastri
Datum	9 - 5
Handtekening	[redacted]

Centrale Commissie Dierproeven

2.

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'.	10700
1.2	Provide the name of the licenced establishment.	University Maastricht
1.3	Provide the title of the project.	Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease

2 Categories

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

- X 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.

Deep brain stimulation (DBS) involves the implantation of stimulating electrodes into specific parts of the brain. DBS is a rapidly emerging area of clinical neuroscience and has evolved to be an effective treatment for patients with Parkinson's disease (PD) [6], essential tremor [7-8], and dystonia [9]. In

patients with severe Tourette syndrome [10] and Obsessive Compulsive Disorder [11-12], DBS can produce therapeutic effects as well. In other neurological and psychiatric disorders, such as Huntington's disease [13], epilepsy [14], depression [15-16], addiction [17-19], and Alzheimer's Disease [20-21], the efficacy of DBS is being explored. The most pronounced beneficial effects have been observed with DBS of the subthalamic nucleus (STN) in PD patients.

Mechanism(s) behind the effects of deep brain stimulation is not fully unraveled:
The mechanisms by which DBS improves the symptoms, has been mostly investigated in PD individuals and animal models with emphasis on the "rate", and "synchronized oscillations" hypotheses [22-23]. In PD, a specific region in the basal ganglia, the STN, exhibits a continuous, abnormal, *burst firing rate* at the single-cell electrophysiological level [24-25] and *synchronized oscillations* with a frequency in the β-range (13–30 Hz) at the level of local field potentials [26], leading to characteristic changes in neuronal firing rates and patterns [27]. At stimulation settings commonly used in clinical practice, DBS decreases spontaneous firing of neuronal populations and drives axonal projections near the electrode [22-23]. These, modulate the pathological activity and replace it with a regular pattern of discharge with intervals of burst activity [28]. However, the exact mechanism(s) by which, DBS normalizes electrical activity in the basal ganglia and exerts beneficial effects on PD symptoms remain unknown. Thus far, research efforts have led to varying outcomes, which cannot be explained by the above-mentioned concept. For instance, based on the rate hypothesis, increased output from the Globus Pallidus internus (GPi) would cause PD symptoms, predicting that STN-DBS suppresses its output [29, 30]. However, STN-DBS is found to increase neuronal activity [31] and glutamate release in the GPi in patients [32]. Furthermore, the relationship between β-oscillations and PD symptoms has been shown to be more complex than initially thought [33]. Therefore, it appears that besides changes in firing rate and pattern of activity, other neuronal processes are involved. A potential mechanism would be a change in the neurochemical properties of the STN neurons, which are known to be exclusively glutamatergic, and its downstream regions. Neurotransmitter identity (type of a neurotransmitter that a neuron produces) of the neurons has been thought to be fixed throughout life, but environmental stimuli can drive behaviorally relevant transmitter switching in the mature brain thorough a recently discovered phenomenon, termed neurotransmitter respecification [2-5]. Our recent research indicates that DBS of the anterior nucleus of the thalamus enhances the number of dopaminergic neurons in the ventral tegmental area, providing evidence for neurotransmitter switching [1].

Neurotransmitter respecification in the mature brain:
Evidence for neurotransmitter respecification in the mature brain has been available for many years but has received surprisingly little attention [34-38]. For instance, early studies in primates demonstrated that the number of gamma-aminobutyric acid (GABA)-ergic neurons in the neocortex of primates is regulated by environmental stimuli [37-38]. Strong evidence for transmitter switching in the mature brain comes from a recent study examining the populations of interneurons in the adult rat hypothalamus, which switched between dopamine and somatostatin expression in response to exposure to short- and long-day photoperiods [3] Notably, in this study the changes in photoperiods are rather extreme than routine changes in day-night cycle that cause distress to the subjects.

Interestingly, in rodent and primate models of PD, lesioning dopaminergic neurons in the substantia nigra pars-compacta (SNc) can lead to the appearance of newly dopaminergic neurons, possibly via neurotransmitter switching from GABA to dopamine [39-40] in an activity-dependent manner [41]. These neurons have similar projection patterns to dopaminergic neurons in the SNc [42-44]. It will be of considerable interest to determine how widespread this process is in the PD brain and how PD symptoms are related to these changes. We hypothesize that STN-DBS triggers phenotype switching of STN neurons from glutamatergic to GABAergic phenotype and/or recruitment of newly dopaminergic neurons from the substantia nigra pars-reticulata (SNr) and/or change in the neuronal expression pattern of GABA/glutamate in the motor cortex and globus pallidus externus (GPe) neurons.

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?

Evidence for neurotransmitter switching has been accumulating steadily, both in the developing nervous system and in the adult brain, with observations of transmitter addition, loss, or replacement of one

transmitter with another. Natural stimuli can drive these changes in transmitter identity, with matching changes in postsynaptic transmitter receptors. Strikingly, they often convert the synapse from excitatory to inhibitory or vice versa, providing a basis for changes in behavior in those cases in which it has been examined. Thus, it has become clear that electrical activity is a factor that can induce transmitter switching, not only during development but in the mature nervous system as well.

We will test the hypothesis that behavioral changes following STN-DBS are mediated by neurotransmitter respecification in the STN and connected regions. These experiments will broaden our understanding of the mechanisms of DBS and will help to improve its current applications and develop new ones.
Our key objectives are to determine:

I. What is the extent of transmitter respecification before and after DBS in an animal model of PD?
II. Which circuits are involved and how? In particular we will address which of the followings will play a role; the local neurons at the target, downstream limbic or motor circuits or the brain region far away from the DBS target.
III. What is the impact of this transmitter respecification on behavioral parameters?

The electrophysiological, anatomical (objective I), behavioral and optogenetics (objectives III) experiments will be performed in our laboratories in Maastricht for approximately 2 years. The molecular biology and Ca2+ imaging experiments will be performed at UCSD (objective II). All techniques are established and running routinely in both laboratories. With regard to optogenetics setup in UM, the setup has been purchase, the safety permissions are obtained.

Our strong scientific back ground, well stablished infrastructures and good collaborators makes the above mentioned aims achievable.
This project is evaluated critically by NWO scientific board and awarded with VENI talent fellowship (2015).

3.3 Relevance

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

At present, thousands of patients with PD have been implanted with DBS electrodes and their number is expected to increase. The success of STN-DBS is based on solid scientific evidence derived from animal research in the 1980's and 1990's [50]. However, the exact mechanism behind the effects of DBS in PD is not well known. In this respect, I have introduced a potential mechanism, which can be used to understand the disease symptoms and in turn optimize and monitor the therapeutic effects.
In this application, we propose an in-depth investigation of the recently discovered mechanism of neuroplasticity, neurotransmitter respecification in PD. From a scientific and clinical point of view, there is a foreseeable possibility of identifying novel mechanisms of brain function that would facilitate the accurate targeting of the stimulation site in the brain and management of the psychiatric, cognitive and motor effects of DBS in patients with neurological disorders. Understanding the essence and extent of neurotransmitter respecification in local and remote neural elements following the application of electrical current will clarify the main components driving the therapeutic benefit, and the mechanisms that facilitate and that work at cross-purposes in patients. These will direct more rational and effective use of DBS and unleash its full therapeutic potential. Our translational DBS group (Prof. Temel) at MUMC will facilitate direct utilization of the scientific outcomes in clinical practice in a relatively short period of time (3-5 years). Neuronal mechanisms behind the therapeutic and side effects of DBS are not entirely known. In fact, many side effects cannot be explained by current knowledge. Investigating the neurotransmitter switching will reveal the changes in different areas and networks. For instance, if it turns out that STN DBS changes the monoaminergic system functionality, it explains why some PD patients suffer from mood/affect disturbances after DBS. This knowledge can be used to adjust the stimulation paradigms to avoid affecting monoaminergic system.
This group, comprised of integrated departments of neuroscience, neurosurgery and neurology, provides a unique team to conduct translational DBS studies. Close collaboration and communication between basic scientists and clinicians in our team has led to a successful implementation of preclinical findings in clinical DBS during the past years.

3.4 Research strategy

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

The project will consist of three independent experiments, related to every objective. Experiments I, II and III involve 3 inter-related sections, which together will provide a multi-level, interdisciplinary investigation of how STN-DBS affects the neurotransmitter identity of cells in the STN and related regions, which are linked to DBS-induced behavioral changes.
We will test the hypothesis that DBS modifies GABA, glutamate and monoamine-dependent behavioral outputs linked to mood, cognition and motor behavior; and that these effects involve the stimulation-derived neurotransmitter respecification in the STN, SNc, SNr, DRN and the neocortex in PD. The microcircuits that are linked to the behavioral effects of the STN-DBS will be selectively challenged (excitation/inhibition) by means of optogenetics to further verify the role of neurotransmitter respecification in mediating the behavioral effects of DBS.
Rats are preferred to mice in neuroscience research for many reasons, including the natural advantages of the rat model, the long history of pioneering development and validation of rat behavioral tasks and readout methods. However, due to following reasons we have to use mice for objectives I, II and III:

1- Genetically modified (Cell specific Cre recombinase) rats are not available and not established scientifically, whilst there are varieties of different mouse lines commercially available besides neurotoxic models.
2- Optogenetics experiments are more feasible in mice than rats.

Although, it should be noted that there is an ongoing rapid advance not only in optogenetics but also in rat genetic tools. This field of neuroscience is expected to continue to grow rapidly. Together with associated enabling technologies, rat optogenetics will likely play a crucial role in contributing to neuroscience research. However, application of optogenetics to the rat system is lagging behind applications to the mouse system by several years (56).

The electrophysiological, anatomical (objectives I), behavioral and optogenetics (objective III) experiments will be performed in our laboratories in Maastricht for approximately 2 years. The molecular biology and Ca^{2+} imaging experiments will be performed at UCSD (objective II), and will take approximately 6 months.

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.

Experiment I: We will identify the neurotransmitter respecification in neural circuit(s)/neuronal cell type(s) in PD and following STN-DBS, by means of combined immunohistochemical (IHC)/stereological and *in vivo* single unit recording methods. Experiments will focus on input and output structures of the STN, which are known to play a role in PD pathology.

The following sets of experiments will be conducted:

Four mice lines will be used; GABA-Cre, TH-Cre, 5-HT-Cre and Glu-Cre.
GABA- Cre mouse line expresses Cre specifically in GABA-ergic cell population.
TH-Cre mouse line expresses Cre specifically in Dopaminergic cell population.
5HT-Cre mouse line expresses Cre specifically in Serotonergic cell population.
Glu-Cre mouse line expresses Cre specifically in Glutamatergic cell population.

Mice will undergo stereotactic surgery and electrode implantation in the STN. Every mouse line will have a PD group which is induced by IP administration of MPTP neurotoxin. After recovery period, combined single cell recording and juxtacellular labeling will be performed under anesthesia to compare the electrophysiological and neurotransmitter finger-prints of the cells in the STN, SNc, SNr and the neocortex following neurotransmitter respecification. Afterwards, the subjects will be perfused transcarially.
We will conduct IHC on postmortem tissues to investigate neurotransmitter respecification in the STN, SNc, SNr, EP and the neocortex in PD and following STN-DBS.

The behavioral and molecular techniques selected for this study are among the ones that have contributed most to our current knowledge about the mechanism of action of DBS and its beneficial/side effects in Parkinson's disease (PD).

Experiment II: The set of experiments to achieve objective 2 will be conducted in UCSD, San Diego,

USA under local regulations.

Experiment III: The microcircuits that are linked to the behavioral effects of the STN-DBS will be selectively challenged (excitation/inhibition) by means of optogenetics to further verify the role of neurotransmitter respecification in mediating the behavioral effects of DBS.

Targeted expression of genes to specific cell types can be robustly achieved with the Cre–loxP system. Recent studies have used 'double-floxed' inverted open reading frame (DIO) viral vectors to achieve high specificity and expression levels [51].
The most commonly used strategy to date for the expression of ChR2 in brain tissue is through viral transduction. Viral vectors driving ChR2 expression can be delivered directly into specific brain regions with robust transduction efficacy and limited tissue damage. Adeno-associated viruses (AAVs) provide extensive spatial spread and high expression levels (52, 55).
The use of a viral DIO construct allows for expression of ChR2 exclusively in the subpopulation of neurons that expresses Cre recombinase. A number of transgenic, BAC and knock-in mouse lines, now commercially available, express Cre in specific populations of excitatory neurons (e.g., CW2, T29-1, T29-2, respectively) or inhibitory interneurons (e.g., PV-Cre). The specificity of ChR2 expression conferred by the DIO construct is very high (52-54). A fluorescent tag, such as mCherry fused to ChR2, allows post-hoc identification and mapping of ChR2-expressing cells for direct assessment of the specificity of expression.
In AAV DIO ChR2-mCherry, two incompatible loxP variants flank an inverted version of ChR2 fused to the fluorescent marker mCherry. In the presence of Cre, a stochastic recombination of either loxP variant takes place27, resulting in the inversion of ChR2-mCherry into the sense direction, followed by expression of the light-activated channels. Cre-dependent expression of light-activated channels or other genes is particularly well suited for targeting expression to cell types that lack identified promoter sequences (51).

The following sets of experiments will be conducted:

i) Mice will undergo stereotactic implantation of a cannula for virus injection and optogenetics probe insertion. Viral vectors will be utilized to induce expression of light-sensitive ion channels in the microcircuits that are identified to undergo neurotransmitter respecification in PD and following DBS. Targeted expression of genes to specific cell types can be robustly achieved with the Cre–loxP system. Recent studies have used 'double-floxed' inverted open reading frame (DIO) viral vectors to achieve high specificity and expression levels [51]. Thereafter these circuits will be challenged by means of optogenetics (instead of DBS) and mice (PD and healthy) and mice will be tested in behavioral settings.

ii) Behavioral experiments (to test PD related behavioral changes) will commence using the open-field test to evaluate locomotion and anxiety and a rotarod will be used to examine motor coordination. Moreover, we will use the elevated zero-maze to measure anxiety-related behavior and the Y-maze test to assess memory. At the end of the experiments, mice will be perfused transcardially.

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 project will consist of 3 inter-related sections, which together will provide a multi-level, interdisciplinary investigation of how STN-DBS affects the neurotransmitter identity of cells in the STN and related regions, which are linked to DBS-induced behavioral changes:

1. To identify the neural circuit(s) and neuronal cell type(s) that undergoes neurotransmitter respecification in a rodent model of PD after STN-DBS (objective.1).
2. To unravel molecular pathways that mediate DBS-derived neurotransmitter respecification (objective.2).
3. To test the hypothesis that behavioral changes following STN-DBS are mediated by neurotransmitter respecification in the STN and associated circuits (objective.3).

The electrophysiological and anatomical experiments will focus on the neurotransmitter identity of the STN, SNc, SNr, entopeduncular nucleus (rodent homologue of the primate GPi) and neocortical neurons

before and after STN-DBS in a rodent model of PD. We will apply STN-DBS using the stimulation parameters that have been shown to be effective in animal models and clinical settings. Control experiments will use electrodes implanted but not stimulated. All experiments will be conducted in three transgenic mouse lines specifically expressing green fluorescent protein (*GFP)* in glutamatergic, GABAergic or dopaminergic neurons. Thereafter these circuits will be challenged by means of optogenetics (instead of DBS) and mice (PD and healthy) will be tested in behavioral settings. Using these animal models will enable me to trace the neurotransmitter identity of the neurons before, during and after DBS. All experiments will be performed in stimulation on/off conditions in a neurotoxin (MPTP)-induced mouse model of PD and corresponding controls.
Based on our finding in experiment I, it might turn out that only one or some of the neurotransmitter systems (GABA, Glutamate, Dopamine and/or Serotonin) has undergone neurotransmitter switching. In this case we will conduct experiments II and III on one or some of the mouse lines. Experiment III is not depended on the out comes from experiment II, which is designed to investigate the mechanisms behind neurotransmitter switching. Experiment III will not be conducted if there is no positive outcome from experiment I in each mouse line. Experiment IV is an independent experiment.

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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	Investigating the extent of transmitter respecification before and after DBS in an animal model of PD.
2	Studying the impact of this transmitter respecification on behavioral parameters in an animal model of PD.
3	
4	
5	
6	
7	
8	
9	
10	

Centrale Commissie Dierproeven

3.

Format
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of deep brain stimulation in Parkinson's Disease
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Ziekte van Parkinson; diepe hersenstimulatie (deep brain stimulation, DBS); neurotransmitter verandering

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

X	Fundamenteel onderzoek
	Translationeel of toegepast onderzoek
☐	Wettelijk vereist onderzoek of routinematige productie
☐	Onderzoek ter bescherming van het milieu in het belang van de gezondheid
☐	Onderzoek gericht op het behoud van de diersoort
☐	Hoger onderwijs of opleiding
☐	Forensisch onderzoek
☐	Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1 Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)

Diepe hersenstimulatie is een veel toegepaste therapie bij patiënten met neurologische en psychiatrische ziektebeelden, zoals de ziekte van Parkinson. Echter de effecten zijn niet optimaal en bijwerkingen kunnen optreden. Wij willen deze therapie verbeteren door onderzoek naar de recent ontdekte verandering in de identiteit van hersencellen a.g.v. elektrische stimulatie.

3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Wetenschappelijke interesse: het definiëren van de hersencircuits die betrokken zijn bij de therapeutische effecten en bijwerkingen van diepe hersenstimulatie (deep brain stimulation, DBS). Zo krijgen we een fundamenteel begrip van hoe DBS de symptomen van de ziekte van Parkinson onderdrukt. Sociaal belang: Een beter begrip van de mechanismen van DBS zal de therapie verbeteren en bijwerkingen verminderen. Daarnaast kan deze nieuwe kennis de weg effenen voor de ontwikkeling van alternatieve en waarschijnlijk minder invasieve therapieën voor neurologische en/of psychiatrische stoornissen.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Er zullen maximaal 800 volwassen muizen voor deze studie gebruikt worden.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Ongerief veroorzaakt door experimentele procedures, zoals chirurgie, meten van hersenactiviteit en gedragstesten. Ongerief veroorzaakt door het opwekken van het Parkinson (PD) model, zoals verminderde eetlust en motivatie.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	De mate van ongerief voor dit onderzoeksproject wordt geclassificeerd als matig. Post-operatieve pijn wordt bestreden met adequate pijnstilling. De stimulatie wordt dusdanig ingesteld dat de dieren er geen hinder van ondervinden.
3.6	Wat is de bestemming van de dieren na afloop?	Aan het eind van de experimenten zullen de dieren diepe anesthesie krijgen voor het meten van de hersenactiviteit, waarna de dieren op een humane manier worden geëuthanaseerd om het brein op cel niveau te onderzoeken.

4 Drie V's

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	We zullen de hypothese testen dat gedragsveranderingen na DBS worden veroorzaakt door neurotransmitter verandering. Deze hypothese kan niet getoetst worden middels humaan onderzoek omdat: 1. Complexe gedragsanalyse van cognitie, geheugen, angst en stemming tijdens de verschillende manieren van diepe hersenstimulatie niet ethisch is bij de mens 2. Het brein niet tot op cel niveau te onderzoeken is bij de mens. 3. De markers die gebruikt worden om de neurotransmitter veranderingen aan te tonen niet veilig in humane studies te gebruiken zijn. Deze hypothese kan tevens niet getest worden wanneer wij computer modellen, celculturen gebruiken, of lagere diersoorten gebruiken, doordat wij de specifieke neurotransmitter productie binnen het hersennetwerk niet kunnen modelleren.
4.2	**Vermindering** Leg uit hoe kan worden	Wij zullen het aantal dieren in dit onderzoek beperken tot een minimum

	verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	door een statistische poweranalyse. Uit onze eigen ervaring en een literatuuroverzicht weten we dat er verlies kan ontstaan van dieren als gevolg van de ziekte van Parkinson en door de operaties. We hebben de poweranalyse hierop aangepast.
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	Het gebruikte diermodel bootst de populatie van patiënten met de ziekte van Parkinson het beste na, is vaak gebruikt en biedt mogelijkheden voor een gedetailleerde analyse van gedrag tot op celniveau welke niet mogelijk is met andere modellen. Vergeleken met andere modellen, kunnen wij door middel van dit diermodel de ziekte van Parkinson relatief snel induceren, welke de tijd van de dieren in het experiment minimaliseert.
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	Alle experimentele procedures worden uitgevoerd door ervaren en geschoolde onderzoekers en verzorgers. Operaties worden uitgevoerd onder algehele anesthesie met adequate pijnbestrijding. Tijdens de postoperatieve herstelperiode worden de dieren zorgvuldig gecontroleerd. De dieren met ziekte van Parkinson worden nauwlettend gevolgd en verzorgd om ernstig ongemak te voorkomen. Tevens zullen we minimaal invasieve benaderingen gebruiken m.b.t de operaties. Ook zullen we de huisvesting en de mate van zorg aanpassen aan de behoeftes van de dieren in de verschillende stadia van de experimenten.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University

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
1	Investigating the extent of transmitter respecification before and after DBS in an animal model of PD.

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 **experimental approach** of this study is to apply Deep Brain Stimulation (DBS) in subthalamic nucleus (STN) of parkinsonian (PD) and sham control mice. Consequently, we will identify the neurotransmitter respecification in neural circuit(s)/neuronal cell type(s) in PD and following STN-DBS, by means of combined immunohistochemical (IHC)/stereological and in vivo single unit recording methods. Experiments will focus on input and output structures of the STN, which are known to play a role in PD pathology.

The electrophysiological and anatomical experiments will focus on the neurotransmitter identity of the STN, SNc, SNr, entopeduncular nucleus (rodent homologue of the primate GPi) and neocortical neurons before and after STN-DBS in a rodent model of PD. We will apply STN-DBS using the stimulation parameters that have been shown to be effective in animal models and clinical settings. Control experiments will use electrodes implanted but not stimulated. All experiments will be conducted in four transgenic mouse lines containing Cre recombinase in specific cell types. Using viral vectors, mice will express fluorescent protein (*GFP or EYFP)* in glutamatergic, GABAergic or monoaminergic neurons. Using these animal models will enable us to trace the neurotransmitter identity (type of neurotransmitter that a given cell produces e.g., serotonergic, dopaminergic etc.,) of the neurons before, during and after DBS. All experiments will be performed in stimulation on/off conditions in a neurotoxin (MPTP)-induced mouse model of PD and corresponding controls (figure 1).

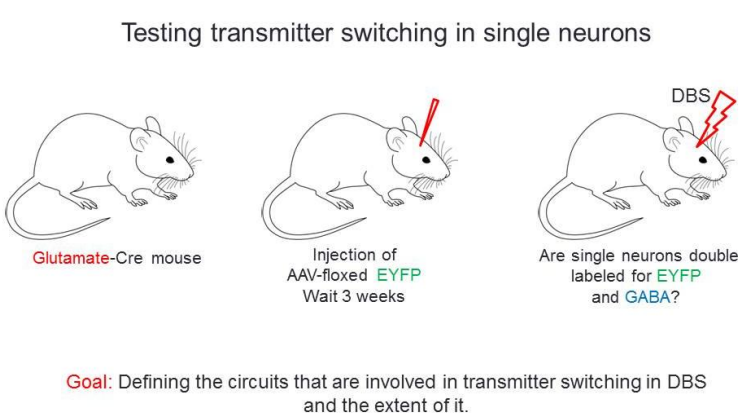

Figure 1: Schematic representation of experiment I. Similar experiments will be conducted in TH, GABA and 5HT-Cre mice lines and corresponding controls in PD and healthy conditions.

The primary outcomes: we will use IHC to investigate neurotransmitter respecification in the STN, SNc, SNr, EP and the neocortex in PD and following STN-DBS. According to the disease pathology and the therapeutic effects of DBS, we expect the following outcomes:
a) alterations in GABA expression in the basal ganglia and related behavioral changes.
b) alterations in dopamine expression in the SNc and SNr and related behavioral changes.
c) alterations in the expression pattern of glutamate/GABA-ergic neurons in the neocortex and related behavioral changes.
d) alterations in GABA expression in the EP and SNr and related behavioral changes.
Double labeling these cell populations with GFP will allow us to show that the neurons have undergone neurotransmitter respecification.

i) Behavioral experiments will commence using different test batteries to evaluate the therapeutic and side effects of DBS in the STN, related to neurotransmitter switching.

ii) The changes in the numbers of neurons expressing different neurotransmitters will be quantified by means of high precision design-based stereology.

iii) Combined single cell recording and juxtacellular labeling will be performed to compare the electrophysiological and neurotransmitter finger-prints of the cells in the STN, SNc, SNr and the neocortex following neurotransmitter respecification.

Table 1. Summary of the different behavioral tests and readout parameters.

Test paradigm	Item	Readout parameter	Discomfort
Object location task	memory	ratio of time spent at object in new versus old location.	Mild discomfort, results in increased anxiety
Skinner box paradigms e.g. reaction time and attention tasks	cognition	reaction time, incorrect responses, correct responses, premature responses	Moderate discomfort, animals will receive restricted feeding regimes or deprivation
Rotarod	locomotion	time spent on rotating cylinder	Mild discomfort, results in increased anxiety
Sucrose	mood	volume of sucrose intake corrected	Mild discomfort,

intake/preference		for animal weight.	results in increased anxiety
Open field	anxiety and locomotion	total distance travelled, average speed and time spent in corners.	Mild discomfort, results in increased anxiety
Elevated zero maze	anxiety	time spent in closed arms	Mild discomfort, results in increased anxiety

Though all animals will undergo a battery of behavioral testing, related to PD symptoms. These tests will be used to assess mood, cognitive and motor functions. The selection of tests may change in the course of the study due to analyses of previous tests. We therefore study STN DBS in different Cre mouse lines to find the behavioral impact of neurotransmitter switching on the behavior. In case of observing discomfort in the animals with one or some the behavioral tasks, an alternative test will be used.
If multiple behavioral tests are suitable and both could answer the research question of interest, we will select the test with the least degree of discomfort for following studies. Cumulative discomfort caused by consequent behavioral testing will be minimized by a wash out period appropriately for the different tests. Each subject will undergo maximum of 6 behavioral tests related to locomotion, mood, anxiety, cognition and memory. The open field test for locomotion and anxiety; the Rotarod test for locomotion; the elevated zero maze for anxiety; the sucrose intake test for mood; object location test for memory and the Skinner box test for cognition. If the open field test reveals the changes in anxiety and locomotion, the elevated zero maze and the Rotarod tests will not be conducted.

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

This study is a chronic DBS experiment and involves DBS, video and electrophysiological recordings, and behavioral tests in freely moving and anesthetized mice. For a summary of the experimental groups see section 2B. The procedures proposed in this study are comparable to those published in literature and previous (un)published studies in which we used similar DBS and behavioral testing paradigms in animal models for neurodegenerative diseases. Based on our previous experience, we estimate a maximal experimental time of 6 months per animal [1-4].

MPTP mouse model of Parkinson's disease

Mice in PD group will be treated with MPTP one week prior to the surgery [5, 6].

Surgery and behavioral experiments
AVV-Floxed EYFP will be infused by means of stereotactic surgery bilaterally in the:
STN in Glu-Cre mice, SNC and SNr in TH-Cre mice, STN and cortex in GABA-Cre mice and the DRN in 5HT-Cre mice. Each mice line will have PD and sham control. Thereafter, mice will undergo stereotactic implantation of DBS electrodes in the STN. All groups will receive 2-4 weeks recovery to allow the Cre recombinase to unpack the EYFP or GFP in order to express the florescent protein in specific cell groups.
Surgical procedures will be conducted under general anesthesia. Sham animal will undergo the same surgical procedure, but are not stimulated through the electrodes.

Subsequently, animals will be subjected to behavioral tests for motor, memory, cognition, mood and anxiety. As mentioned in section 2A, the choice of behavioral test may vary in course of the study. Behavioral tests will be performed in both DBS *on* and *off* conditions (for maximally 6 months of behavioral testing). Experimental groups consist of animals that receive high (130 Hz) frequency DBS in stimulation paradigms, which are used in our previous PD research [2, 4].

Single cell electrophysiology and juxtacellular recording
At the end of the behavioral experiments mice will be subjected to single cell electrophysiology under anesthesia (terminal experiment).

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

To minimize the number of animals, we will consider and apply published studies, previous studies by our group and a power analyses. Moreover, this study is designed in several levels and phases. Based on this phase-designed approach we will test our hypothesis step by step in smaller badges of animals but not at once. This approach will prevent unnecessary/extra experiments.

B. The animals

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

Animals

These experiments require cell specific Cre containing adult mice, as these animals are used routinely in previous optogenetic studies [7].

Gender

We will only use male mice, since previous studies have shown that the oestrogen cycle can interfere with brain neurochemistry. Sex hormones have been implicated in neurite outgrowth, synaptogenesis, dendritic branching, myelination and other important mechanisms of neural plasticity. Recent evidences from animal experiments and human studies report interactions between sex hormones and the dominant neurotransmitters, such as serotonin, dopamine, GABA and glutamate. Accumulating data during physiological and pathological conditions and discuss currently conceptualized theories on how sex hormones potentially trigger neuroplasticity changes through these four neurochemical systems. Many brain regions have been demonstrated to express high densities for estrogen- and progesterone receptors, such as the amygdala, the hypothalamus, and the hippocampus. These changes have been linked to differences in behavior, neurochemical patterns and hippocampal structure to a changing hormonal environment. Physiologically occurring hormonal transition periods changes in sex hormones influence functional connectivity, neurotransmission and brain structure in vivo [8-12].

In this study we will use 10-12 weeks old mice. Based on our experience, mice at this age are more suitable for electrode/cannula implantation. In older mice (or rats) the sutures position change on the skull. This makes it difficult to navigate stereotactic implantation. Besides, jaw muscles grow towards midline by aging and therefore leave a little space for electrode/cannula construct.

Number of animals

We estimation on the total number of 25 animals/group is based on our previous experience with behavioral studies and a literature review [6].

We estimate a maximum number of 400 animals for this study.

Table 1: description of the experimental groups.

Experimental groups for Glu-Cre line	*Number of mice/group*
Naïve Sham	25
MPTP Sham	25
Naïve electrical DBS	25
MPTP electrical DBS	25
Experimental groups for GABA-Cre line	
Naïve Sham	25
MPTP Sham	25
Naïve electrical DBS	25
MPTP electrical DBS	25
Experimental groups for TH-Cre line	
Naïve Sham	25
MPTP Sham	25
Naïve electrical DBS	28
MPTP electrical DBS	25
Experimental groups for 5HT-Cre line	
Naïve Sham	25

MPTP Sham	25
Naïve electrical DBS	25
MPTP electrical DBS	25
Total	400

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.

Replacement

The primary aim of this study is to test the hypothesis that behavioral changes following STN-DBS are mediated by neurotransmitter respecification in the STN and connected regions.

These aims cannot be achieved by use of in vitro experiments or computer modelling, because these models do not allow for analysis of behavior and do not represent the organization of a complex neuronal network in a complex biological system such as the brain. In previous studies [13, 14] we have successfully shown that by using DBS in several rat models of neurodegenerative diseases, we can model the therapeutic and behavioral side effects of DBS. The current study can also not be performed in humans because of the following: 1. Complex behavioral analysis of cognition, memory, anxiety and mood during different stimulation paradigms compared to sham operation is not ethical in humans 2. This project involve using Cre construct in specific cell types is not possible in human subjects. 3. The approach in this study requires using AVV viral vectors, which again restricted in human studies.

Reduction

We will limit the number of animals in this study to a minimum by using a power analysis. The power analyses are based on our primary outcome measure: behavioral improvement. By decreasing group-size we will under power this study which may lead to falls results. Besides, we expect variable responses to DBS as well as the neurotoxin that will be applied to induce PD in mice. Current group size will allow us to subdivide the subject based on their response and understand the neurobiological bases of their responses. In addition, applying a "phased design" is taken into account to reduce the number of animals used throughout the experiments. Specifically; if turns out that only one or some of the neurotransmitter systems (GABA, Glutamate, Dopamine and/or Serotonin) has undergone neurotransmitter switching in experiment "I", we will conduct experiments II and III on one or some of the mouse lines. We will take the following measurements to reduce loss of animals: Most drop out is expected for mice with PD in compare to the healthy subjects. For this reason, we will conduct a daily welfare evaluation and tracking with special attention on PD mice; in particular their nutrition and body weight.

Refinement

We choose the MPTP animal model for PD, since animals by this model exert behavioral phenotype of the PD, therefore are likely to show PD-related neurotransmitter switching. Based on our literature review, MPTP is the best method to induce PD in mice [15-17]. As a matter of fact, choosing the animal model, with resembles the human condition most, results in shorter and less complex behavioral experiments. Moreover, MPTP can be administered intraperitoneally. This will thus decrease the time that the animals

are in experiment, unlike surgical administration, which adds few weeks to the experiment. Additionally, we have put extra efforts to design the experiment as such that cause less distress to the animals. In particular, the best and most up to date surgical procedures, electrode construct, drug administration methods and behavioral tests are planned. Moreover, we know from our own previous published/unpublished studies that which readout measures are more representative and useful. For instance; following observation of certain behavioral phenotype (e.g. impulsivity, depressive like behavior etc.,), what sort of analysis should be conducted. Knowing these, will shorten the duration of the experiments. The proposed experiment is dealing with both healthy and PD animals. An increased discomfort is expected for mice with PD symptoms in compare to the healthy subjects. For this reason, we will conduct a daily welfare evaluation and tracking with special attention on PD mice; in particular their nutrition and body weight. Finally, we will adapt the accommodation and the care to the need of the animals at the different time stages during the experiment.

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

Animals will be under general anesthesia during surgical procedures and will receive appropriate analgesics during the post-operative recovery period. The electrode implantation is similar to the implantation of stimulation electrodes in the clinic, performed under local anesthesia. These patients experience minor pain; therefore there is no need for analgesics. In laboratory animals, this is done under general anesthesia. Animals will receive Pre and post-operative analgesia. In addition, preoperative, local anesthetic will be injected at the site of incision. In experiment, if a mouse shows sign of pain, distress, infection or inflammation, it will be treated with analgesics, antibiotics or anti-inflammatory medications. We will prevent pain and discomfort by monitoring the animals during behavioral experiments. The injections and behavioral test will be conducted according to standard guidelines. Similarly, if animals show pain during any of the experimental procedures described, they will receive analgesics. The persons involved in this experiment possess a strong background of laboratory animal welfare experiences to minimize animal suffering, pain or fear.

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 concept of neurotransmitter switching in adult mammals is very new. Besides us, there is only one group (UCSD, San Diego, USA), that conducts research on this subject, whom we will collaborate with in this project. When combining neurotransmitter switching concept and DBS, there will be no possibility for duplication, meaning that we are aware of this filed and will not do an experiment, which has been done/being done by others. Notably, the novelty and originality of this project has been evaluated recently by the scientific board of NWO and granted with VENI research fellowship.

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.

All animals will be housed socially, unless fighting or damaging the electrode construct. The cages and water will be renewed once a week; and the weight of the animals will be measured and written down in a laboratory book.

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.

General anesthesia will be used for surgical procedures. Animals will be monitored daily during the experimental procedures stated in section 2A and will be checked for signs of pain or discomfort. Standard analgesic will be applied to relief suffering e.g. during the post-surgical recovery period.

I. Other aspects compromising the welfare of the animals

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

A possible adverse effect which might occur is loss of an electrode construct. Mice with PD symptoms might face adverse effects in particular their nutrition and body weight.
Viral tools used in this study are not infectious [7, 18]. The viral tools are applied locally in a very small quantity. Thus, do not induce any immune reaction.

Explain why these effects may emerge.

The electrode construct is fixed on the skull of the animal using dental cement. In the postoperative period the head skin of the animal will heal and grow around the electrode construct. However infection may still occur. Special care will be taken for mice with PD. However, due to lack of dopaminergic neurotransmission, some animals might have less motivation for food and thus lose weight.

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

Animals will be visually inspected daily during experiments. During recovery period after surgery, mice will be inspected several times a day and body weight will be measured every day. Recovery boost gel will be administered if animals are not gaining weight. Prophylactic antibiotic will be administered. In case of adverse effects, the experiment will be halted and the animals will be treated accordingly.

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.

In case of electrode detachment, severe illness or tumor growth human end point will be applied. More complications are expected for mice with PD symptoms in compare to the healthy subjects. Lesioning dopaminergic system will affect their nutrition and body weight. Neurotoxin induced PD model in this study is not considered as a progressive model. Majority of PD symptoms start to disappear slowly after several months. This is due to regeneration and sprouting of dopaminergic neurons. These experiments will be conducted before regeneration starts. Greater side effects are expected to appear during the first couple of weeks after neurotoxin infusion.

Lesioning the DA cells affects the animals' ability to ambulate and perform normal body functions, and these potential effects on health and well-being mandate additional steps to ensure humane animal care and use: if clinical signs of PD disables the animal to keep normal nutrition requirements despite special nursing and care, which are indicative for moderate exceeding discomfort, humane endpoint will be applied. Scoring on general impression (awareness, gait, performing species specific behavior, body condition and body composition, posture, fur/skin appearance, fascial expression), clinical signs of PD, body weight, hydration state and performance in behavior tasks will be used to define the humane endpoint. If those symptoms persists longer than 36 hours (despite treatment), mice will be euthanized. An animal welfare score will be used to evaluate the animal wellbeing.

Indicate the likely incidence.

We estimate the likely incidence at maximally 10%. However, the pilot and proof of principle studies described in section 2A are carried out to reduce this incidence in both this study and for the studies described in appendix 2 and 3.

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').

The expected level of discomfort is mild for all animals due to stereotactic surgery for implantation of the electrode construct, virus injection and behavioral testing. We consider the discomfort caused by the PD model to be moderate. Cumulative discomfort caused by these experiments is expected to be 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.

At the end of the experiments, all animals will be sacrificed to isolate the brain and perform histologic analysis. Histologic analysis of the brain is needed to check for the correct electrode localization and to study the neuroanatomical effects of DBS and correlate this to behavioral outcome.

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

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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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
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: Studying the impact of this transmitter respecification on behavioral parameters in an animal model of PD.

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 **experimental approach** of this study is to apply optogenetic DBS in the networks that involve in stimulation-derived neurotransmitter respecification in parkinsonian (PD) and sham control mice. Consequently, we will test the hypothesis that DBS modifies GABA, glutamate and monoamine-dependent behavioral outputs linked to mood, cognition and motor behavior; and that these effects involve the stimulation-derived neurotransmitter respecification in the STN, SNc, SNr and the neocortex in PD. The microcircuits that are linked to the behavioral effects of the STN-DBS (which will be defined in experiment I) will be selectively challenged (excitation/inhibition) by means of optogenetics to further verify the role of neurotransmitter respecification in mediating the behavioral effects of DBS. Targeted expression of genes to specific cell types can be robustly achieved with the Cre–loxP system. Recent studies have used 'double-floxed' inverted open reading frame (DIO) viral vectors to achieve high specificity and expression levels. Therefore, specific cell types will be modulated by means of optogenetic neuromodulation.

We will apply optogenetic neuromodulation using the stimulation parameters that have been shown to be effective in animal models [1, 2]. Optogenetic stimulation of the STN in rodent models of PD has shown to be effective in modulating cell firing pattern, local field potentials and oscillatory pattern of activity [1, 2]. Control experiments will use cannula implanted but not stimulated. The experiments will be conducted in four transgenic mouse lines with containing Cre recombinase construct in specific cell types. Using viral vectors, mice will express fluorescent protein (*GFP or EYFP) and channelrhodopsin* in glutamatergic, GABAergic or monoaminergic neurons. All experiments will be performed in stimulation

on/off conditions in a neurotoxin (MPTP)-induced mouse model of PD and corresponding controls (figure 1).

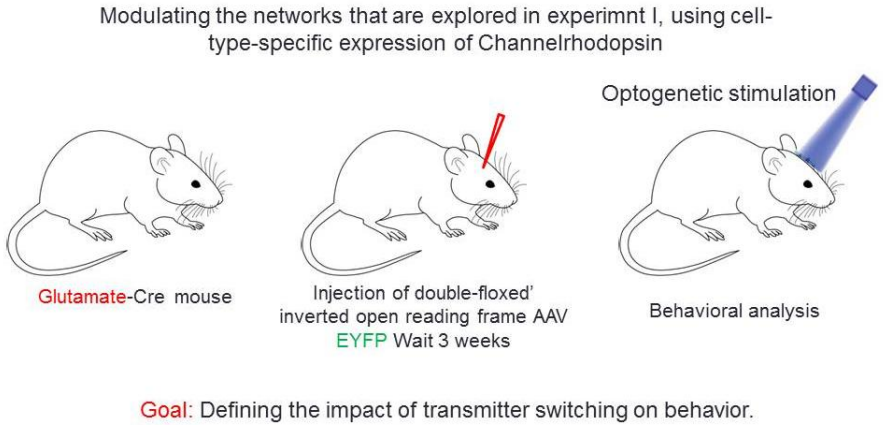

Figure 1: Schematic representation of experiment II. same experiment will be conducted in TH, GABA and 5HT-Cre mice lines and corresponding controls in PD and healthy mice.

The primary outcomes: we will use IHC to verify neurotransmitter switching in the STN, SNc, SNr, EP and the neocortex in PD and following optogenetic neuromodulation. According to the disease pathology and the therapeutic effects of DBS, we expect more beneficial outcomes (eg., improved dyskinesia) and less side effect (eg., depressive-like behavior) with optogenetic neuromodulation of specific cell types. The therapeutic and side effects will be evaluated using behavioral test battery.

i) Behavioral experiments will commence using different test batteries to evaluate the therapeutic and side effects of DBS, related to neurotransmitter switching.

ii) The changes in the numbers of neurons expressing different neurotransmitters will be quantified by means of high precision design-based stereology.

iii) Combined single cell recording and juxtacellular labeling will be performed to compare the electrophysiological and neurotransmitter finger-prints of the cells in the STN, SNc, SNr and the neocortex following neurotransmitter respecification.

Table 1. Summary of the different behavioral tests and readout parameters.

Test paradigm	Item	Readout parameter	Discomfort
Object location task	memory	ratio of time spent at object in new versus old location.	Mild discomfort, results in increased anxiety
Skinner box paradigms e.g. reaction time and attention tasks	cognition	reaction time, incorrect responses, correct responses, premature responses	Moderate discomfort, animals will receive restricted feeding regimes or deprivation
Rotarod	locomotion	time spent on rotating cylinder	Mild discomfort, results in increased anxiety
Sucrose intake/preference	mood	volume of sucrose intake corrected for animal weight.	Mild discomfort, results in increased anxiety
Open field	anxiety	total distance travelled, average	Mild discomfort,

	and locomotion	speed and time spent in corners.	results in increased anxiety
Elevated zero maze	anxiety	time spent in closed arms	Mild discomfort, results in increased anxiety

Though all animals will undergo a battery of behavioral testing, related to PD symptoms. These tests will be used to assess mood, cognitive and motor functions. The selection of tests may change in the course of the study due to analyses of previous tests. We therefore study optogenetics DBS in different Cre mouse lines to find the behavioral impact of neurotransmitter switching on the behavior. In case of observing discomfort in the animals with one or some the behavioral tasks, an alternative test will be used. If multiple behavioral tests are suitable and both could answer the research question of interest, we will select the test with the least degree of discomfort for following studies. Cumulative discomfort caused by consequent behavioral testing will be minimized by a wash out period appropriately for the different tests. Each subject will undergo maximum of 6 behavioral tests related to locomotion, mood, anxiety, cognition and memory. The open field test for locomotion and anxiety; the Rotarod test for locomotion; the elevated zero maze for anxiety; the sucrose intake test for mood; object location test for memory and the Skinner box test for cognition. If the open field test reveals the changes in anxiety and locomotion, the elevated zero maze and the Rotarod tests will not be conducted.

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

This study is a chronic DBS experiment and involves optogenetic DBS, video and electrophysiological recordings and behavioral tests in freely moving and anesthetized mice. For a summary of the experimental groups see section 2B. The procedures proposed in this study are comparable to those published in literature and previous (un)published studies in which we used similar DBS and behavioral testing paradigms in animal models for neurodegenerative diseases. Based on our previous experience, we estimate a maximal experimental time of 6 months per animal [3-6].

MPTP mouse model of Parkinson's disease

Mice in PD group will be treated with MPTP one week prior to the surgery [7, 8].

Surgery and behavioral experiments
Double-floxed inverted open reading frame viral vector will be infused to achieve targeted expression of genes to specific cell types by means of stereotactic surgery bilaterally in the:
STN in Glu-Cre mice, SNC and SNr in TH-Cre mice, STN and cortex in GABA-Cre mice and the DRN in 5HT-Cre mice. Each mice line will have PD and sham control. Thereafter, mice will undergo stereotactic implantation of guide cannula (to insert optogenetic probe). All groups will receive 2 - 4 weeks recovery to allow the Cre recombinase to unpack the EYFP or GFP in order to express the florescent protein in specific cell groups.
Surgical procedures will be conducted under general anesthesia. Sham animal will undergo the same surgical procedure, but are not stimulated through the cannulas.

Subsequently, animals will be subjected to behavioral tests for motor, memory, cognition, mood and anxiety. As mentioned in section 2A, the choice of behavioral test may vary in course of the study. Behavioral tests will be performed in both DBS *on* and *off* conditions (for maximally 6 months of behavioral testing). Experimental groups consist of animals that receive optogenetics DBS in stimulation paradigms, which are used in previous studies [3-6].

Single cell electrophysiology and juxtacellular recording
At the end of the behavioral experiments mice will be subjected to single cell electrophysiology under anesthesia (terminal experiment) (see SOP nr 5). Subsequently, animals are euthanized by either perfusion or decapitation as required for follow-up histological analysis of the brain.

Describe which statistical methods have been used and which other considerations have been taken into

account to minimize the number of animals.

To minimize the number of animals, we will consider and apply published studies, previous studies by our group and a power analyses. Moreover, this study is designed in several levels and phases. Based on this phase-designed approach we will test our hypothesis step by step in smaller badges of animals but not at once. This approach will prevent unnecessary/extra experiments.

B. The animals

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

Animals
We estimation on the total number of 25 animals/group is based on our previous experience with behavioral studies and a literature review [8].

Gender
We will only use male mice, since previous studies have shown that the oestrogen cycle can interfere with brain neurochemistry. Sex hormones have been implicated in neurite outgrowth, synaptogenesis, dendritic branching, myelination and other important mechanisms of neural plasticity. Recent evidences from animal experiments and human studies report interactions between sex hormones and the dominant neurotransmitters, such as serotonin, dopamine, GABA and glutamate. Accumulating data during physiological and pathological conditions and discuss currently conceptualized theories on how sex hormones potentially trigger neuroplasticity changes through these four neurochemical systems. Many brain regions have been demonstrated to express high densities for estrogen- and progesterone receptors, such as the amygdala, the hypothalamus, and the hippocampus. These changes have been linked to differences in behavior, neurochemical patterns and hippocampal structure to a changing hormonal environment. Physiologically occurring hormonal transition periods changes in sex hormones influence functional connectivity, neurotransmission and brain structure in vivo [9-13].
In this study we will use 10-12 weeks old mice. Based on our experience, mice at this age are more suitable for electrode/cannula implantation. In older mice (or rats) the sutures position change on the skull. This makes it difficult to navigate stereotactic implantation. Besides, jaw muscles grow towards midline by aging and therefore leave a little space for electrode/cannula construct.

Number of animals
The estimation on the total number of animals is based on our previous experience with DBS studies and a literature review. We expect a group size of animals 25 mice [8].
We estimate a maximum number of 400 animals for this study.
Table 1: description of the experimental groups.

Experimental groups for Glu-Cre line	*Number of mice/group*
Naïve Sham	25
MPTP Sham	25
Naïve optogenetic neuromodulation	25
MPTP optogenetic neuromodulation	25
Experimental groups for GABA-Cre line	
Naïve Sham	25
MPTP Sham	25
Naïve optogenetic neuromodulation	25
MPTP optogenetic neuromodulation	25
Experimental groups for TH-Cre line	
Naïve Sham	25
MPTP Sham	25
Naïve optogenetic neuromodulation	25
MPTP optogenetic neuromodulation	25
Experimental groups for 5HT-Cre line	
Naïve Sham	25
MPTP Sham	25

Naïve optogenetic neuromodulation	25
MPTP optogenetic neuromodulation	25
Total	400

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.

Replacement

The primary aim of this study is to test the hypothesis that behavioral changes following STN-DBS are mediated by neurotransmitter respecification in the STN and connected regions.

These aims cannot be achieved by use of in vitro experiments or computer modelling, because these models do not allow for analysis of behavior and do not represent the organization of a complex neuronal network in a complex biological system such as the brain. In previous studies [14, 15] we have successfully shown that by using DBS in several rat models of neurodegenerative diseases, we can model the therapeutic and behavioral side effects of DBS. The current study can also not be performed in humans because of the following: 1. Complex behavioral analysis of cognition, memory, anxiety and mood during different stimulation paradigms compared to sham operation is not ethical in humans 2. This project involve using Cre construct in specific cell types is not possible in human subjects. 3. The approach in this study requires using AVV viral vectors, which again restricted in human studies.

Reduction

We will limit the number of animals in this study to a minimum by using a power analysis. The power analyses are based on our primary outcome measure: behavioral improvement. By decreasing group-size we will under power this study which may lead to falls results. Besides, we expect variable responses to DBS as well as the neurotoxin that will be applied to induce PD in mice. Current group size will allow us to subdivide the subject based on their response and understand the neurobiological bases of their responses. In addition, applying a "phased design" is taken into account to reduce the number of animals used throughout the experiments. Specifically; if turns out that only one or some of the neurotransmitter systems (GABA, Glutamate, Dopamine and/or Serotonin) has undergone neurotransmitter switching in experiment "I", we will conduct experiments II and III on one or some of the mouse lines. We will take the following measurements to reduce loss of animals: Most drop out is expected for mice with PD in compare to the healthy subjects. For this reason, we will conduct a daily welfare evaluation and tracking with special attention on PD mice; in particular their nutrition and body weight.

Refinement

We choose the MPTP animal model for PD, since animals by this model exert behavioral phenotype of the PD, therefore are likely to show PD-related neurotransmitter switching. Based on our literature review, MPTP is the best method to induce PD in mice [16-18]. As a matter of fact, choosing the animal model, with resembles the human condition most, results in shorter and less complex behavioral experiments. Moreover, MPTP can be administered intraperitoneally. This will thus decrease the time that the animals are in experiment, unlike surgical administration, which adds few weeks to the experiment. Additionally, we have put extra efforts to design the experiment as such that cause less distress to the animals. In particular, the best and most up to date surgical procedures, cannula construct, drug administration

methods and behavioral tests are planned. Moreover, we know from our own previous unpublished study that which readout measures are more representative and useful. For instance; following observation of certain behavioral phenotype (e.g. impulsivity, depressive like behavior etc.,), what sort of analysis should be conducted. Knowing these, will shorten the duration of the experiments. The proposed experiment is dealing with both healthy and PD animals. An increased discomfort is expected for mice with PD symptoms in compare to the healthy subjects. For this reason, we will conduct a daily welfare evaluation and tracking with special attention on PD mice; in particular their nutrition and body weight. Finally, we will adapt the accommodation and the care to the need of the animals at the different time stages during the experiment.

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

Animals will be under general anesthesia during surgical procedures and will receive appropriate analgesics during the post-operative recovery period. The cannula implantation is similar to the implantation of cannula in the clinic, performed under local anesthesia. These patients experience minor pain; therefore there is no need for analgesics. In laboratory animals, this is done under general anesthesia. Animals will receive Pre and post-operative analgesia. In addition, preoperative, local anesthetic will be injected at the site of incision. In experiment, if a mouse shows sign of pain, distress, infection or inflammation, it will be treated with analgesics, antibiotics or anti-inflammatory medications. We will prevent pain and discomfort by monitoring the animals during behavioral experiments. The injections and behavioral test will be conducted according to standard guidelines. Similarly, if animals show pain during any of the experimental procedures described, they will receive analgesics. The persons involved in this experiment possess a strong background of laboratory animal welfare experiences to minimize animal suffering, pain or fear.

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 concept of neurotransmitter switching in adult mammals is very new. Besides us, there is only one group (UCSD, San Diego, USA), that conducts research on this subject, whom we will collaborate with in this project. When combining neurotransmitter switching concept and DBS, there will be no possibility for duplication, meaning that we are aware of this filed and will not do an experiment, which has been done/being done by others. Notably, the novelty and originality of this project has been evaluated recently by the scientific board of NWO and granted with VENI research fellowship.

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.

All animals will be housed socially, unless fighting or damaging the cannula construct.The cages and water will be renewed once a week; and the weight of the animals will be measured and written down in a laboratory book.

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.

General anesthesia will be used for surgical procedures. Animals will be monitored daily during the experimental procedures stated in section 2A and will be checked for signs of pain or discomfort. Standard analgesic will be applied to relief suffering e.g. during the post-surgical recovery period.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?
A possible adverse effect which might occur is loss of an implanted construct. Mice with PD symptoms might face adverse effects in particular their nutrition and body weight.
Viral tools used in this study are not infectious [1, 2]. The viral tools are applied locally in very small quantity. Thus, do not induce any immune reaction.

Explain why these effects may emerge.

The cannula construct is fixed on the skull of the animal using dental cement. In the postoperative period the head skin of the animal will heal and grow around the construct. However infection may still occur. Special care will be taken for mice with PD. However, due to lack of dopaminergic neurotransmission, some animals might have less motivation for food and thus lose weight.

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

Animals will be visually inspected daily during experiments. During recovery period after surgery, mice will be inspected several times a day and body wait will be measured every day. Recovery boost gel will be administered if animals are not gaining weight. Prophylactic antibiotic will be administered. In case of adverse effects, the experiment will be halted and the animals will be treated accordingly.

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.

In case of cannula detachment, severe illness or tumor growth, humane end point will be applied. More complications are expected for mice with PD symptoms in compare to the healthy subjects. Lesioning dopaminergic system will affect their nutrition and body weight. Neurotoxin induced PD model in this study is not considered as a progressive model. Majority of PD symptoms start to disappear slowly after several months. This is due to regeneration and sprouting of dopaminergic neurons. These experiments will be conducted before regeneration starts. Greater side effects are expected to appear during the first couple of weeks after neurotoxin infusion.
Lesioning the DA cells affects the animals' ability to ambulate and perform normal body functions, and these potential effects on health and well-being mandate additional steps to ensure humane animal care and use: if clinical signs of PD disables the animal to keep normal nutrition requirements despite special

nursing and care, which are indicative for moderate exceeding discomfort, humane endpoint will be applied. Scoring on general impression (awareness, gait, performing species specific behavior, body condition and body composition, posture, fur/skin appearance, fascial expression), clinical signs of PD, body weight, hydration state and performance in behavior tasks will be used to define the humane endpoint. If those symptoms persists longer than 36 hours (despite treatment), mice will be euthanized. An animal welfare score will be used to evaluate the animal wellbeing.

Indicate the likely incidence.

We estimate the likely incidence at maximally 10%. However, the pilot and proof of principle studies described in section 2A are carried out to reduce this incidence in both this study and for the studies described in appendix 2 and 3.

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').

The expected level of discomfort is mild for all animals due to stereotactic surgery for implantation of the cannula, virus injection and behavioral testing. We consider the discomfort caused by the PD model to be moderate. Cumulative discomfort caused by these experiments is expected to be 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.

At the end of the experiments, all animals will be sacrificed to isolate the brain and perform histologic analysis. Histologic analysis of the brain is needed to check for the correct cannula localization and to study the neuroanatomical effects of DBS and correlate this to behavioral outcome.

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

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6.

DEC-advies PV-2015-014-[redacted]

A. Algemene gegevens over de procedure

1. Aanvraagnummer; 2015-014

   Titel van het project; *Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease.*

2. Titel van de NTS; *Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease.*

3. Type aanvraag:

   X nieuwe aanvraag projectvergunning

   ☐ wijziging van vergunning met nummer

4. Contactgegevens DEC:

   - naam DEC; DEC-UM
   - telefoonnummer contactpersoon; [redacted]
   - mailadres contactpersoon; [redacted]

5. Adviestraject (data dd-mm-jjjj):

   X ontvangen door DEC; 08-03-2016

   ☐ aanvraag compleet

   X in vergadering besproken; 18-03-2016

   ☐ anderszins behandeld

   ☐ termijnonderbreking van 24-03-2016 tot 30-03-2016 /12-04-2016 tot 13-04-2016

   ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen

   ☐ aanpassing aanvraag

   ☐ advies aan CCD

6. Eventueel horen van aanvrager **NVT**

7. Correspondentie met de aanvrager:

- Datum 24-03-2016

Strekking van de vragen:

1) **3.1:** U geeft aan te werken met 5-HT cre muizen. Desalniettemin ligt uw focus nergens op de raphe nucleus, waar de 5-HT cellen zich bevinden. Kunt u toelichten waarom u deze muizen wil gebruiken?
2) **3.4.2:** De muisexperimenten zijn helder opgeschreven en ook de modellen die gebruikt gaan worden zijn state-of-the-art. De DEC-UM vraagt zich echter af hoe de vraagstellingen 1, 2, en 3 worden vertaald met de literatuur (bv. wat is er bekend over DBS in muizen) – en hoe vertaalt zich de overstap naar ratten, beschreven in deelvraag 4?
3) **3.4.2:** Zijn de muismodellen geschikt om acute effecten van DBS op te pikken met deze reporter cellen?
4) **3.4.2:** In hoeverre is het bekend dat DBS acuut gezien al gunstige effecten heeft die aannemelijkerwijze niet zullen samenhangen met neurotransmitter switchen?
5) **3.4.3:** Er is een goed verband tussen de verschillende experimentele vraagstellingen. Deelvraag 1 bepaalt welk neurotransmitter systeem (of systemen) verder bestudeerd gaan worden in 2 en 3. Alle genetische muismodellen zijn voorhanden, maar hoeven dus niet allemaal te worden gebruikt. Aim 3 zal niet worden uitgevoerd als onder 1 geen positief resultaat wordt behaald. Ook hier geldt; deelvraag 4 is een onafhankelijk experiment en de vraag is: is dit een aparte studie, omdat dit in een ander diermodel wordt uitgevoerd en omdat dit los staat van deelvragen 1, 2 en 3?

3.4.4-appendix 1:

6) Er worden heel veel tests aangehaald waarvan max. 5 per dier worden gebruikt. Zijn dit altijd dezelfde tests en op basis van welke criteria worden deze gekozen? Kunt u misschien al specifieker aangeven welke tests u wilt gaan doen?

7) Er worden radiotracers gebruikt voor PET-CT scans. Zijn er pilotdata dat deze met voldoende resolutie/ specificiteit iets kunnen zeggen? Is dit haalbaar (resolutie PET is ~180um)?

8) De DEC-UM wenst een betere onderbouwing voor de gevraagde aantallen, alsmede een overzicht van de diergroepen, experimenten en berekening voor de aantallen.

9) In het hele document spreekt u van “human endpoints”. De DEC hoopt dat u 'Humane endpoints' bedoelt?

10) PD komt vrijwel niet voor bij mensen jonger dan 50 jaar. Reden voor het alleen gebruiken van mannen is dat vrouwelijke hormonen de hersenen veranderen. Waarom gebruikt u niet oudere ratten (vanaf 15-20 weken in plaats van 10-13 weken) en dan beide geslachten, zodat u een betere weerspiegeling krijgt van de patiëntengroep?
Als DBS een effect heeft op verandering van neurotransmitters, dan willen we dat toch voor zowel mannen als vrouwen weten?
U heeft niets vermeld over de leeftijd van de gebruikte muizen. Wellicht kunt u hier ook specificeren vanaf welke leeftijd u muizen in experiment wilt nemen en kunt u heroverwegen om zowel mannen als vrouwen te gaan gebruiken.

11) De DEC-UM vraagt zich af of er in de microcircuits veranderingen optreden met de leeftijd?

12) F: Accommodation and care: hierin schrijft u dat u niet voldoet aan Annex III of the Directive 2010/63/EU. U bedoelt waarschijnlijk dat u dit wel doet?

13) 3.4.4-appendix 3: Dit is een PD model in ratten, terwijl de rest van de experimenten in muizen gebeurt. Deelvraag 3 (MPTP model) gebruikt ook een PD model maar dan in muis. Zou dit niet gecombineerd kunnen worden?

- Datum antwoord 30-03-2016
- Niet alle vragen zijn naar tevredenheid beantwoord en de DEC-UM heeft dd. 12-04-2016 op nieuw een aantal vragen gesteld.
- De vragen zijn dd. 13-04-2016 naar tevredenheid beantwoord en hebben geleid tot aanpassing van de aanvraag.

8. Eventuele adviezen door experts (niet lid van de DEC) **NVT**

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet); **JA**
2. De aanvraag betreft een **nieuwe aanvraag.**
3. De DEC is competent om hierover te adviseren; **JA**
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering **NVT**.

C. Beoordeling (inhoud):

1. Het project is:

   X uit wetenschappelijk oogpunt verantwoord
   ☐ uit onderwijskundig oogpunt verantwoord
   ☐ uit het oogpunt van productiedoeleinden verantwoord
   ☐ wettelijk vereist

2. De in de aanvraag aangekruiste doelcategorieën zijn in overeenstemming met de hoofddoelstellingen.
3. De DEC onderschrijft het belang van de doelstelling. Het wordt ingeschat als

een substantieel belang.

4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project: **JA.**
5. Er is sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren. De keuze hiervoor is voldoende wetenschappelijk onderbouwd. **NVT**
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd: **JA.**
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.**
8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de vermindering van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat. Vermindering wordt ondermeer verkregen door een powerberekening te hanteren, gebaseerd op resultaten uit het verleden en door een gefaseerd design toe te passen.

9. Het project is in overeenstemming met de vereiste van de verfijning van dierproeven en het project is zo opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Er is geen sprake van belangwekkende milieueffecten. Verfijning krijgt fraai vorm ondermeer door transgene diermodellen te kiezen die de ziekte van Parkinson het meest betrouwbaar weergeven en door minimaal invasieve benaderingen te hanteren.

10. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd

D. Ethische afweging

De DEC-UM heeft het project "*Neurotransmitter switching: A novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease"* bestudeerd. Het behelst terminale experimenten met matig ongerief gedurende 5 jaar met in totaal 800 transgene muizen. Anderzijds onderschrijft de DEC-UM de intrinsieke waarde van het dier.
De DEC-UM is overtuigd van de wetenschappelijke waarde en het uiteindelijk maatschappelijke belang van het voorgestelde onderzoeksproject.
Het project beoogt een beter begrip op te leveren van de werking van Deep Brain Stimulation (DBS) bij de behandeling van de ziekte van Parkinson (PD). Het gaat om het ontrafelen van de achterliggende neuronale mechanismen. Derhalve is het terecht

geclassificeerd als fundamenteel onderzoek. Aangezien DBS op dit moment wordt ingezet bij de behandeling van PD en wordt onderzocht bij andere aan de hersenen gerelateerde aandoeningen, kunnen de resultaten te zijner tijd ook klinisch relevant blijken. Het onderzoek kan zowel een beter begrip opleveren van therapeutische effecten als van eventuele bijwerkingen.
Resultaten van dit onderzoek zijn belangrijk voor PD patiënten en op termijn wellicht ook voor personen die lijden aan andere neurologische en psychiatrische ziekten en hun naasten. De DEC-UM acht derhalve de doelstelling van dit onderzoek van substantieel belang.
De opzet van het onderzoeksproject is helder, logisch en navolgbaar. De doelstellingen van de diverse onderdelen en de stapsgewijze aanpak, zijn overtuigend. De aanvrager beschikt over de benodigde wetenschappelijke kennis en technische expertise. Er is geen sprake van duplicatie. De gewenste uitkomsten zijn relevant in het licht van de overkoepelende vraagstelling en zijn ook haalbaar.
In de gekozen strategie, technieken en diermodellen wordt op bevredigende wijze tegemoet gekomen aan de vereisten op het gebied van vervanging, vermindering en verfijning. De DEC-UM is ervan overtuigd dat er geen alternatieven zijn, waardoor deze dierproef met minder muizen zou kunnen worden uitgevoerd, dan wel het gebruik van levende dieren zou kunnen worden vermeden.
Op grond van deze argumenten acht de DEC-UM in haar ethische afweging het belang van project 2015-014 "*Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease"* van zwaarder gewicht dan de voorziene schade (matig ongerief en dood) voor de maximaal 800 betrokken dieren. De DEC-UM beschouwt de voorgestelde dierproeven derhalve als ethisch gerechtvaardigd en voorziet het projectvoorstel "*Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease"* van een positief advies.

E. Advies

1. Advies aan de CCD

   X De DEC adviseert de vergunning te verlenen

2. Het uitgebrachte advies is gebaseerd op consensus.

Op grond van alle voor de afweging relevante argumenten komt de DEC-UM tot de conclusie dat dit onderzoek ethisch toelaatbaar is.

Centrale Commissie Dierproeven

7.

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Onze referentie
Aanvraagnummer
AVD107002016543

Bijlagen
2

Datum 12 mei 2016
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 10 mei 2016.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD107002016543. 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. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u 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 voldoen, 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.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10700
Naam instelling of organisatie:	Universiteit Maastricht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	50169181
Straat en huisnummer:	Minderboedersberg 4-6
Postbus:	616
Postcode en plaats:	6200 MD MAASTRICHT
IBAN:	NL04INGB0679510168
Tenaamstelling van het rekeningnummer:	Universiteit Maastricht

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]

Gegevens verantwoordelijke uitvoering proces

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 maart 2016
Geplande einddatum:	1 maart 2021
Titel project:	Neurotransmitter switching a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease
Titel niet-technische samenvatting:	Neurotransmitter switching a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease
Naam DEC:	DEC Um
Postadres DEC:	Postbus 616, 6200 MD Maastricht
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 1.187,-
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:	Maastricht
Datum:	9 mei 2016

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Universiteit Maastricht
[redacted]
Postbus 616
6200 MD MAASTRICHT

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD107002016543

Bijlagen
2

Datum 12 mei 2016
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 12 mei 2016
Vervaldatum: 11 juni 2016
Factuurnummer: 16700543

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD107002016543	€ 1.187,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL41RBOS 056.999.6317 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

8.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Universiteit Maastricht
[redacted]
Minderbroedersberg 4-6
Postbus 616
6200MD Maastricht

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
AVD107002016543

Uw referentie

Bijlagen

Datum 7 juni 2016
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 11 mei 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Neurotransmitter switching: a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease' met aanvraagnummer AVD107002016543. 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:

- In de bijlage dierproeven geeft u aan 25 dieren/groep te gebruiken en een maximum aantal 400 dieren voor elke dierproef nodig te hebben. Echter ontbreekt de uitleg van de berekening van de 400 dieren en de beschrijving van de experimentele groepen. We verzoeken u om de berekeningen te verduidelijken, en het aantal groepen te benoemen en te onderbouwen.

- De titel van uw Niet-technische samenvatting is in het Engels doorgegeven. Omdat de NTS voor het publiek bedoeld is, moet de informatie die daarin komt in het Nederlands zijn. We verzoeken u om de titel in het Nederlands in te vullen en een nieuwe NTS naar ons toe te sturen.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Gebruik hierbij het formulier dat u bij deze brief krijgt indien u uw antwoord per post verstuurt. Om uw aanvraag in de eerstkomende vergadering te kunnen bespreken verzoeken we u vriendelijk om uiterlijk maandag 13 juni 2016 uw antwoord naar ons toe te sturen.

Wanneer een beslissing
De behandeling van uw aanvraag wordt 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.

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

11.

> Retouradres Postbus 20401 2500 EK Den Haag

Universiteit Maastricht
[redacted]
Postbus 616
6200 MD MAASTRICHT

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD107002016543

Bijlagen
1

Datum 27 juni 2016
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 10 mei 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neurotransmitter switching a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease " met aanvraagnummer AVD107002016543. Wij hebben uw aanvraag beoordeeld.

Op 16 juni en 28 juni 2016 heeft u uw aanvraag aangevuld. U heeft de titel van uw Niet-technische samenvatting naar het Nederlands vertaald en aangepast.

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. De voorwaarde betreffende het afstemmen van de go/no-go momenten met de IvD is gesteld in het kader van de 3V's, om te voorkomen dat dat dieren onnodig worden gebruikt in het geval dat de experimenten niet de verwachte resultaten opleveren. De algemene voorwaarde betreffende artikel 10, lid 1a van de wet wordt gesteld bij vergunningen met een langere looptijd. Dit om te voldoen aan datgene wat volgt uit dit artikel. U kunt met uw project "Neurotransmitter switching a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease " starten. De vergunning wordt afgegeven van 30 juni 2016 tot en met 1 maart 2021.

Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC Um gevoegd. Dit advies is opgesteld op 10 mei 2016. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet. Wij hebben de DEC om aanvullende informatie gevraagd. Op 1 juni 2016 heeft de DEC gereageerd op onze vragen. De DEC heeft het antwoord van de aanvrager op een door de DEC gestelde vragen naar de CCD gestuurd.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie, nemen wij over, inclusief de daaraan ten grondslag liggende motivering. In aanvulling op het advies van de DEC stelt de CCD twee algemene voorwaarden.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving 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.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze:

[redacted]
Algemeen Secretaris

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

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Universiteit Maastricht
Adres:	Postbus 616
Postcode en plaats:	6200 MD MAASTRICHT
Deelnemersnummer:	10700

deze projectvergunning voor het tijdvak 30 juni 2016 tot en met 1 maart 2021, voor het project "Neurotransmitter switching a novel mechanism behind the therapeutic and side effects of DBS in Parkinson's Disease " met aanvraagnummer AVD107002016543, volgens advies van Dierexperimentencommissie DEC Um. De functie van de verantwoordelijk onderzoeker is Professor Neurochirurgie. Voor de uitvoering van het project is Postdoc verantwoordelijk.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 10 mei 2016
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 11 mei 2016;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 27 juni 2016;
c Advies van dierexperimentencommissie d.d. 10 mei 2016, ontvangen op 11 mei 2016.
d De aanvullingen op uw aanvraag, ontvangen op 16 juni en 28 juni 2016

Naam proef	**Diersoort/ Stam**	**Aantal dieren**	**Ernst**	**Opmerkingen**
3.4.4.1 Investigating the extent of transmitter respecification before and after DBS in an animal model of PD.	Muizen (Mus musculus) / Glu-Cre; TH-Cre; GABA-Cre; 5HT-Cre.	400	100,00 % Matig	
3.4.4.2 Studying the impact of this transmitter respecification on behavioral parameters in an animal model of PD.	Muizen (Mus musculus) / Glu-Cre; TH-Cre; GABA-Cre; 5HT-Cre.	400	100,00 % Matig	

Voorwaarden

Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen

In dit project worden dierproeven toegepast waarbij en wordt daarom voorzien van beoordeling achteraf. Deze beoordeling zal uiterlijk plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst de dierproeven conform de vergunning waren.
De vergunning wordt verleend onder de voorwaarde dat 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.

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 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 13d 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 13c 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.
De Minister heeft vrijstelling ontheffing verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.