	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**
	NTS2016542								
1	Aanvraagformulier				x		x		
2	Projectvoorstel oud			x					
3	Niet-technische samenvatting oud			x					
4	Bijlage beschrijving dierproeven 1 oud			x					
5	Bijlage beschrijving dierproeven 2 oud			x					
6	Bijlage beschrijving dierproeven 2 oud			x					
7	Bijlage beschrijving dierproeven 2 oud				x		x		
8	DEC-advies				x		x		
9	Ontvangstbevestiging				x		x		
10	Antwoord onderzoeker op gestelde vragen DEC			x					
11	Verzoek aanvulling aanvraag				x		x		
12	Reactie aanvulling aanvraag				x		x		
13	Aanvraagformulier herzien				x		x		
14	Projectvoorstel herzien			x					
15	Niet-technische samenvatting herzien	x		x					
16	Bijlage beschrijving dierproeven 1 herzien			x					
17	Bijlage beschrijving dierproeven 2 herzien			x					
18	Bijlage beschrijving dierproeven 3 herzien			x					
19	Bijlage beschrijving dierproeven 4 herzien			x					
20	Adviesnota CCD		x						x
21	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]
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

☐ Dhr. ☒ Mw.

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	[redacted]
Functie	[redacted]
Plaats	Maastric[redacted]
Datum	9 - 5
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 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.	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.

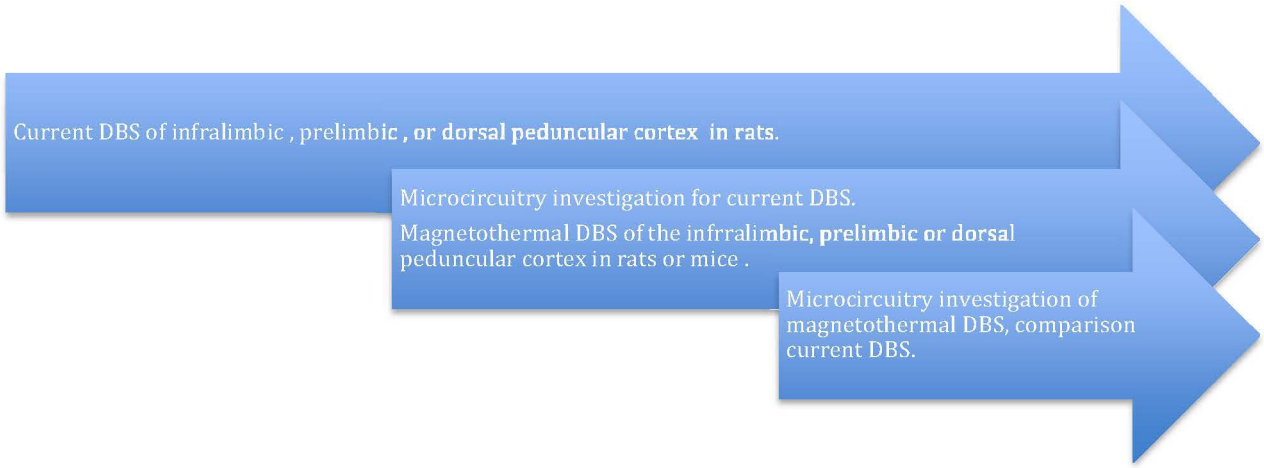

Fig. 1 **Time schedule of our 'magnetothermal and current deep brain stimulation in experimental depression' research project**

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	
6	
7	

8	
9	
10	

References

1. Bejjani, B.P., P. Damier, I. Arnulf, L. Thivard, A.M. Bonnet, D. Dormont, etal.Y. Agid, *Transient acute depression induced by high-frequency deep-brain stimulation.* N Engl J Med, 1999. **340**(19): p. 1476-80.
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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	Magnetothermale diepe hersenstimulatie (DBS) in een diermodel voor depressie
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Magnetothermale diepe hersenstimulatie (DBS); depressie; diermodel

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)

Ernstige depressie is een ziektebeeld dat veel sociaaleconomische gevolgen met zich meebrengt. De behandeling van deze aandoening is niet voor iedereen succesvol en nog verre van optimaal.

Een recent toegepaste behandeling is diepe hersenstimulatie (DBS) met tot op heden nog maar matig succes. In ons project onderzoeken wij een nieuwe verfijnde techniek van DBS genaamd *'magnetothermale diepe hersenstimulatie'* voor depressie in diermodellen. Voor deze verfijnde techniek is, in tegenstelling tot de huidige DBS techniek, het plaatsen van electroden in het brein niet meer nodig. Met deze techniek kunnen we specifieke groepen hersencellen stimuleren, draadloos en op afstand. Dit zorgt voor een

		meer nauwkeurig gecontroleerde hersenactivatie dan dat nu met de huidige DBS techniek mogelijk is. Met deze nieuwe techniek en een juiste onderzoeksstrategie hopen wij meer inzicht te krijgen in het onderliggende mechanisme van depressie. Tevens hopen we een nieuwe therapie voor patiënten met therapieresistente depressie en voor mogelijk andere stemmingsstoornissen of neuronale aandoeningen te kunnen ontwikkelen.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Wetenschappelijk belang: inzicht krijgen in het onderliggende mechanisme van depressie en het ontwikkelen van een nieuwe therapie voor patiënten met depressie die niet reageren op de huidige behandeling. Maatschappelijk belang: het ontwikkelen van een nieuwe, minder invasieve en beter werkende, therapie voor patiënten met depressie die niet reageren op de huidige behandeling en het hierdoor kunnen terugdringen van de sociaal economische gevolgen van depressie.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Wij gebruiken maximaal 1950 ratten.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Alle ratten worden voor het opwekken van depressie blootgesteld aan chronische stress en ondergaan operaties. DBS zelf veroorzaakt mild ongerief. Een deel van de ratten zal gedragstesten ondergaan, een ander deel van de ratten ondergaat metingen van hersenactiviteit tijdens DBS.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Tijdens ons onderzoek gebruiken we chronische stress voor het opwekken van depressief gedrag. Dit veroorzaakt ernstig ongerief. De controlegroep ervaart dit ernstige ongerief niet. De gedragstesten en het meten van hersenactivatie veroorzaken mild tot matig ongerief.
3.6	Wat is de bestemming van de dieren na afloop?	Aan het eind van de experimenten ondergaat elke dier euthanasie. Het brein wordt intact gelaten voor aanvullende analyses.

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.	Ons onderzoek kan niet met alleen cellen of computermodellen verricht worden aangezien het in deze modellen niet mogelijk is om depressief gedrag te analyseren. Ons onderzoek kan ook niet in mensen worden uitgevoerd aangezien magnetothermale DBS eerst nog verder moet worden ontwikkeld en omdat het meten van hersenactivatie met zeer belastende technieken nodig is.

4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Voor vermindering van het aantal ratten hebben wij een literatuurstudie verricht en maken wij gebruik van een diermodel voor depressie dat reeds is gebruikt in ons laboratorium. Voorafgaand aan elk experiment verrichten wij statistische power-berekeningen om het aantal ratten tot een minimum te beperken. We zullen ons eerst bekwamen in de techniek van 'Magnetothermale DBS' aan de Massachusetts Institute of Technology (MIT), waardoor het aantal gebruikte ratten tot een minimum kan worden beperkt.
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.	Met het chronisch stress model, bestaande uit herhaalde toepassingen van oncontroleerbare en onvoorspelbare stress, zijn we in staat om een reeks van gedragsveranderingen te bewerkstelligen lijkend op depressief gedrag in de mens. Dit is met andere modellen niet goed mogelijk en voor onderzoek van depressie is dit daarom het meest verfijnde en meest representatieve diermodel voor depressie. Het model is gebaseerd op klinisch bewijs dat stressvolle levensgebeurtenissen die het risico op depressieve episoden verhogen meestal van chronische aard zijn. Voor verfijning van de technieken van 'magnetothermische DBS', microdialyse en elektrofysiologie, volgen we trainingen aan respectievelijk MIT (USA) en de Universiteit van Oxford (U.K.).
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	Alle ratten krijgen na de operatie een herstelperiode met indien nodig aanvullende pijnstilling. Tijdens de experimenten worden de ratten dagelijks gecontroleerd. We implementeren een welzijn score lijst en zullen hiermee zover mogelijk het ongerief tot een minimum reduceren. Bij buitensporig ongerief wordt de rat uit het experiment gehaald en geëuthanaseerd.

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: 1 Type of animal procedure: 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 penducular 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 penducular 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 stainings. These techniques enables 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, dorsopeduncular=15; n=45) and their sham controls (n=10). And the naïve group CUS- with current DBS (n=30) 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 140 rats.

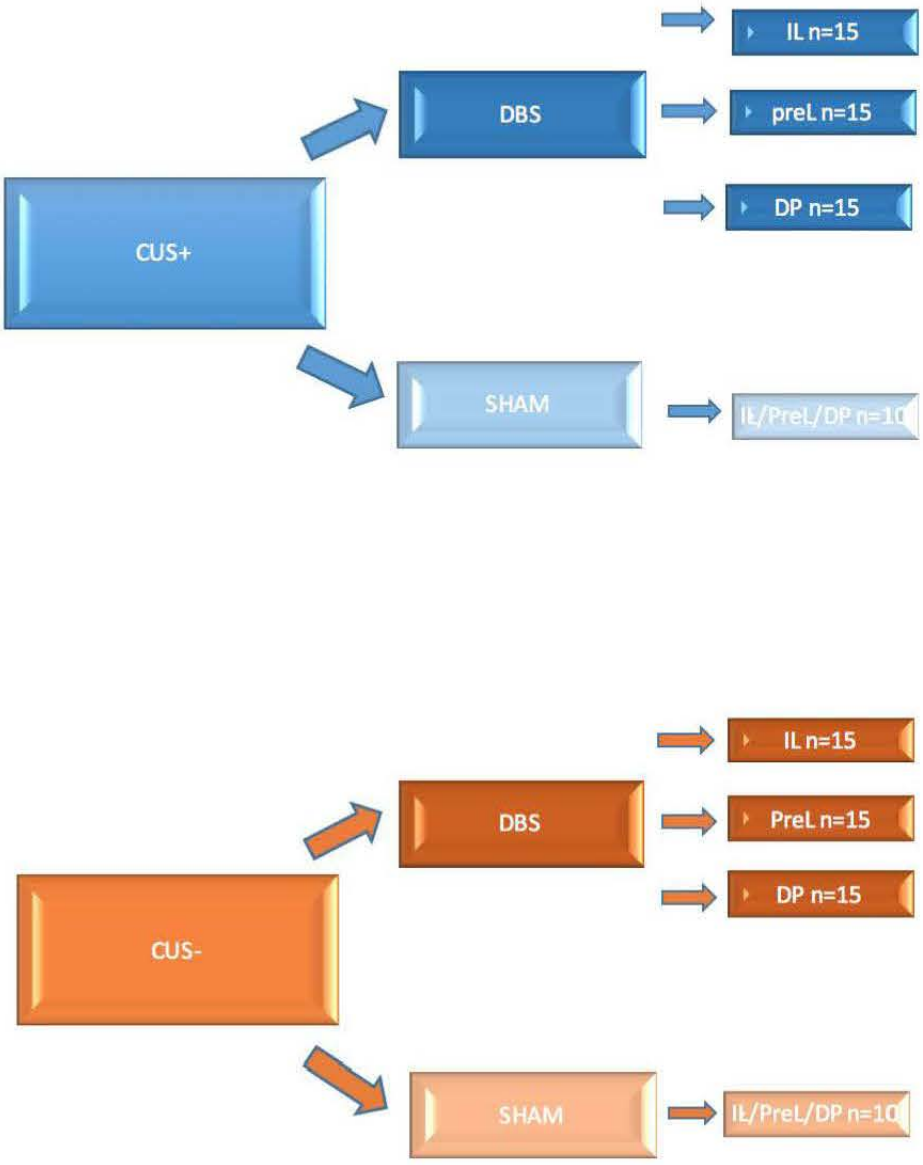

Figure 1. Subdivision of groups for Current DBS.

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 stainings. 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 transcardially 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 follwed 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 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

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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
		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 penducular 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 microdialyses 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 penducular 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 (presumably three). 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, dorsopeduncular; 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.).

Electrophysiology: the experimental group CUS+ with current 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 current DBS (n=135) and their sham controls (n=30)(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 830 rats.

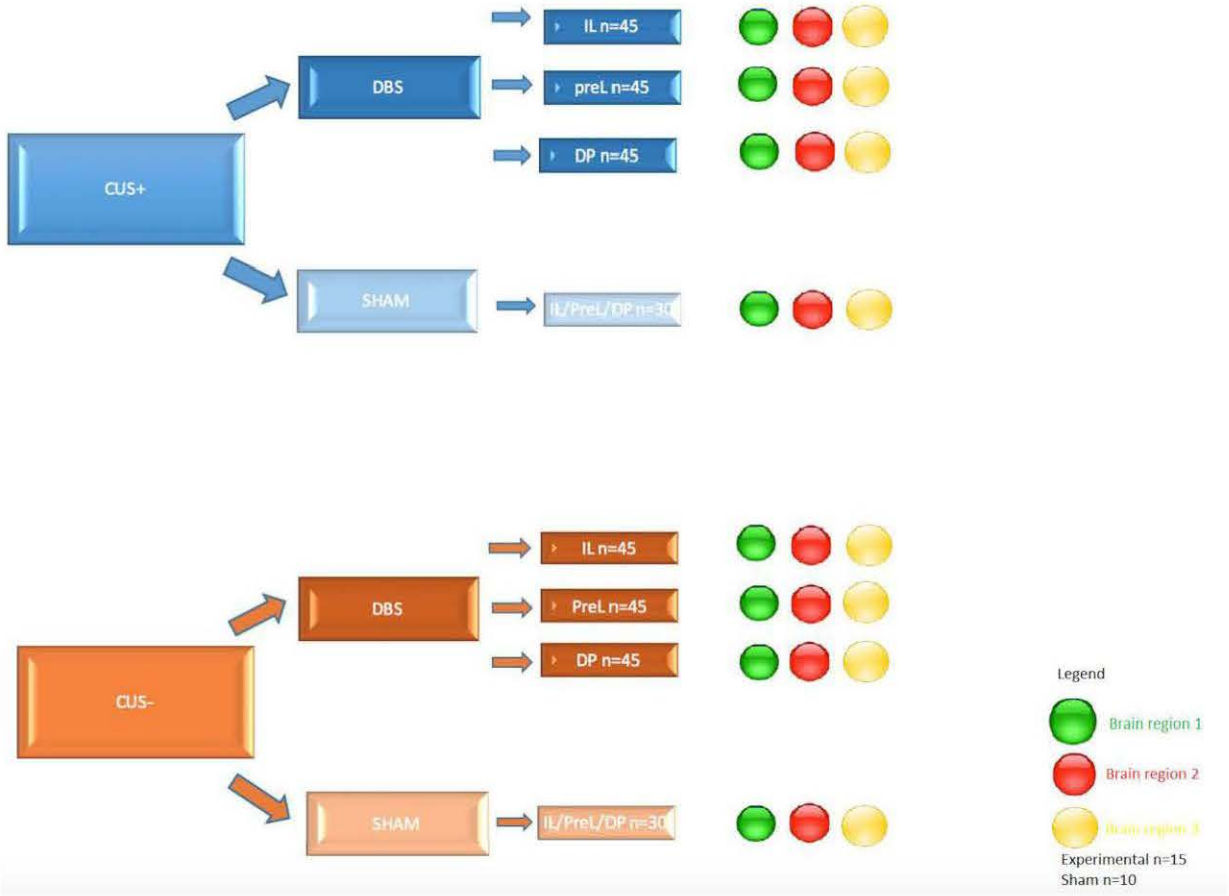

Figure 1. Subdivision of groups for Current DBS and additional microdialysis (n=15) or electrophysiology (n=15), together with a sham control group (n=10). Each brain region indicated with a green, red or yellow circle, will either be a microdialysis experiment or an electrophysiological experiment.

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 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 simultaneously 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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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 prefrontalcortex (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 stainings 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 microcircuits 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 dorsopeduncular 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 stainings 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 anti-depressant effects as 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 magneothermal 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, dorsopeduncular=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 140 rats.

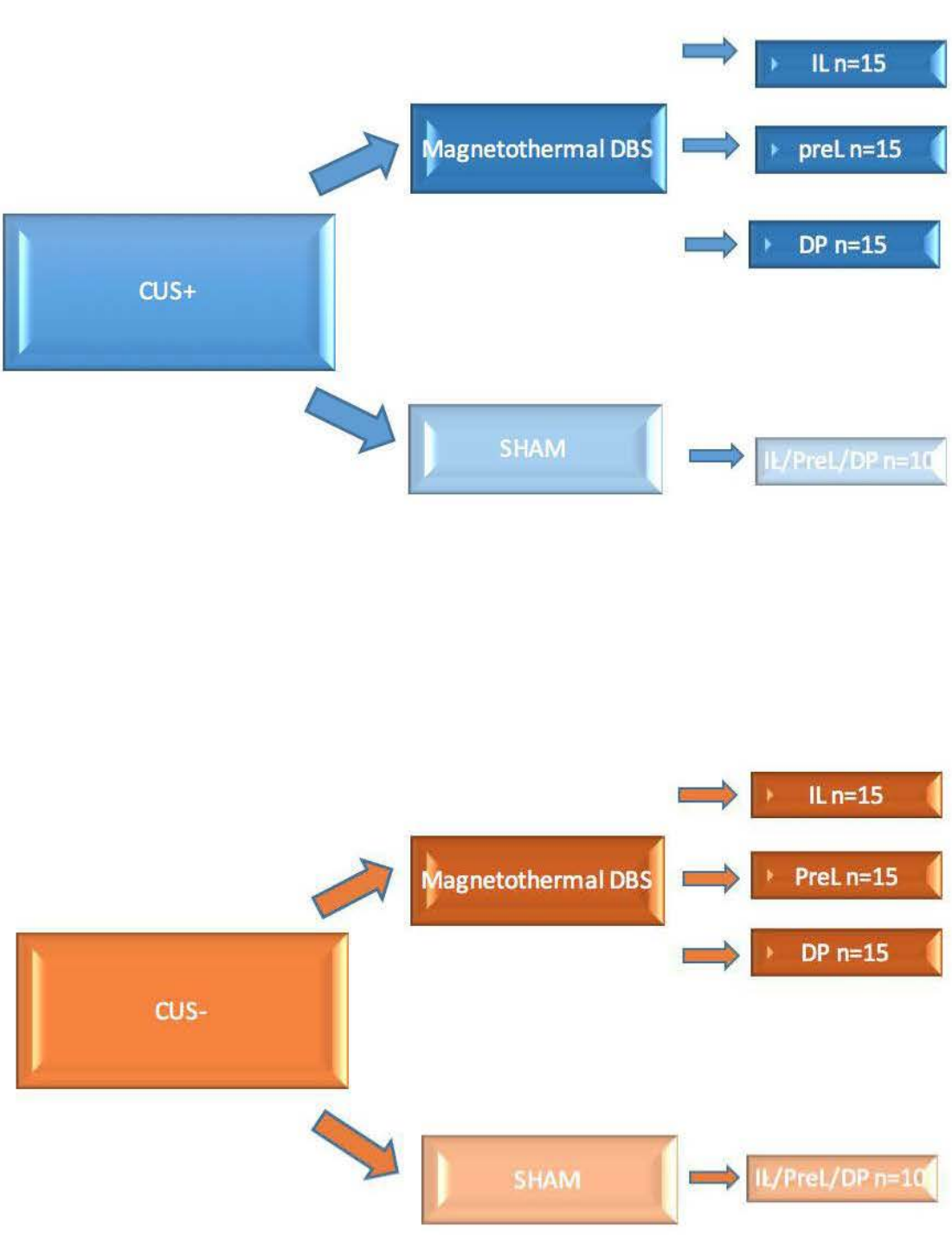

Figure 1. Subdivision of groups for magnetothermal DBS.

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 follwed 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.
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10. Willner, P., *Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS.* Neuropsychobiology, 2005. **52**(2): p. 90-110.
11. 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.