	Inventaris Wob-verzoek W16-09S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2015327								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel				x			x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1				x			x	
5	Bijlage beschrijving dierproeven 2				x			x	
6	Bijlage beschrijving dierproeven 3				x			x	
7	DEC-advies				x		x	x	
8	Ontvangstbevestiging				x		x	x	
9	Advies CCD		x						x
10	Beschikking en vergunning				x		x	x	

AVD 1030020155327

27 NOV 2015

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☑ Ja > *Vul uw deelnemernummer in* 10300
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	Stichting Katholieke Universiteit Nijmegen
Naam van de portefeuillehouder of diens gemachtigde	Instantie voor Dierenwelzijn
KvK-nummer	4 1 0 5 5 6 2 9

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

Straat en huisnummer	Geert Grooteplein 10
Postbus	9101, [redacted]
Postcode en plaats	6500HB Nijmegen
IBAN	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer	UMC St Radboud

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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

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

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

(Titel) Naam en voorletters	[redacted] ☐ Dhr. ☐ Mw.
Functie	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	instantievoordierenwelzijn@radboudumc.nl

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: 25-12-2015
	Einddatum: 25-12-2020
3.2 Wat is de titel van het project?	Neural correlates of post-traumatic stress disorder: natural resilience as key for intervention.
3.3 Wat is de titel van de niet-technische samenvatting?	Onderzoek naar de hersenkenmerken die beschermen tegen post-traumatische stress stoornis.
3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC: RU DEC
	Postadres: Postbus 9101, 6500 HB Nijmegen [redacted]
	E-mailadres: [redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

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

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

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

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ■ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☐ Melding Machtiging
- ☒ DEC-advies, factuurinformatie

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	[redacted]
Functie	[redacted]
Plaats	Nijmegen
Datum	25-11-2015
Handtekening	[redacted]

Form
Project proposal

- This form should be used to write the project propsal of 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'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	Neural correlates of post-traumatic stress disorder: [redacted]

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 animal health or welfare dier
[] 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.

Post-traumatic stress disorder (PTSD) is a debilitating disease which typically develops after a person is exposed to a traumatic event. It is characterized by a variety of symptoms such as flashbacks, hyperarousal, and insomnia, which severely deteriorate quality of life (1,2). Current estimates are that PTSD affects ~8% of the population (3), but numbers seem to be increasing rapidly (4). A variety of medications is currently used to 'treat' PTSD, but since the neural basis of PTSD is still largely unknown, treatments are symptomatic, only effective for fewer than half the patients (5), and side effects and residual symptoms following treatment are rule rather than exception.

Although substantial effort has been put into the elucidation of PTSD pathophysiology (6), successes have been limited due to the enormous heterogeneity of typical patient cohorts, caused by differences in genetic and environmental factors, trauma exposure, medication use (7), and comorbidity (8). Moreover, data from patients is generally obtained *following* the diagnosis of PTSD, making it practically impossible to determine whether the observed differences between psychiatric patients and healthy controls are related to respective causes (i.e., vulnerability) or consequences (i.e., symptoms) of PTSD. Delineation of these factors would advance diagnosis of the disorder and treatment of the correct symptoms, preventing escalation. Furthermore, one is clearly limited in the methods of studying the human brain, as invasive measures are highly undesirable.

Recent neuroscientific findings have indicated that the brain is organized as a set of functional networks (9), which are often reciprocally connected and represent unique brain functions. Imbalance in the activity and connectivity of these networks has been proposed to underlie complex mental disorders such as PTSD (10-15). We hypothesize that PTSD may result from a *chronic imbalance in neural network function* in terms of activity and connectivity (either present at baseline, occurring in response to trauma exposure, or during recovery) in which emotional (i.e., salience) processing overrules cognitive function. However, the exact mechanistic underpinnings of this chronic imbalance in neural network function and when it exactly occurs during PTSD development, is currently unclear. This disturbed neural network balance could either be present (to a certain extent) during baseline, i.e., before any trauma exposure has happened (Figure 1, scenario 1), develop upon trauma exposure (Figure 1, scenario 2), or following (inadequate) recovery of the traumatic experience (Figure 1, scenario 3). It is important to distinguish these scenario's, since each of them would have different practical implications for health care. Identification of of individuals at risk even before trauma-exposure (scenario 1) might argue against their fitness for highly stressful occupations, or for their immediate treatment when trauma-exposure has occurred, and thereby contribute to the prevention of PTSD. The identification of markers for abnormal response to the trauma (scenario 2) would allow for the close monitoring and

identification of the pathology-related neural changes in trauma-exposed individuals, opening the opportunity for early diagnosis and intervention. Lastly, characterization of the pathology-related changes (scenario 3) could be used as marker for diagnosis in a later stage of PTSD, reducing the frequency of misdiagnoses and providing a lead for new treatment options.

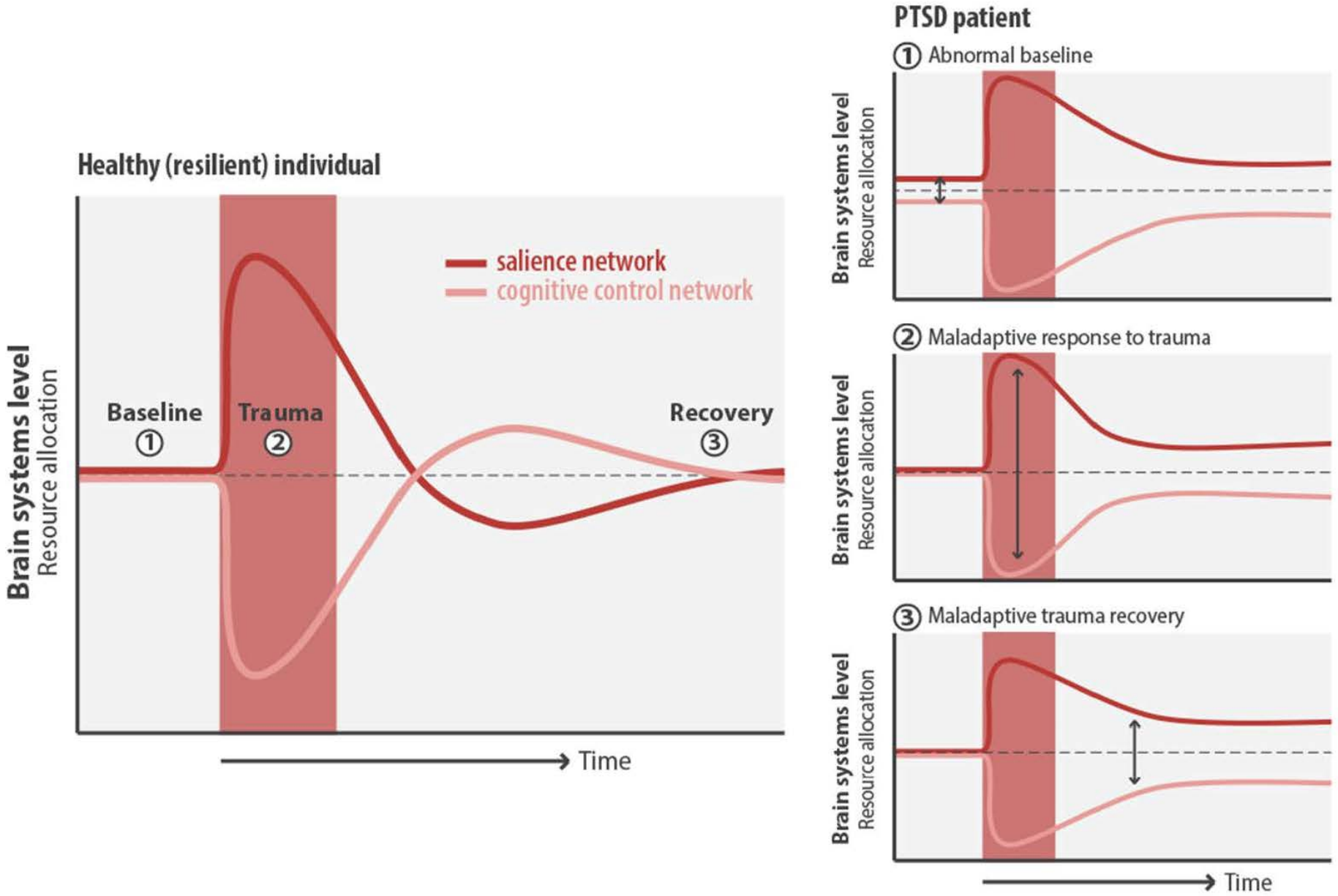
Healthy (resilient) individual
salience network
cognitive control network
Brain systems level
Resource allocation
Baseline
①
Trauma
②
Recovery
③
Time
PTSD patient
① Abnormal baseline
② Maladaptive response to trauma
③ Maladaptive trauma recovery
Brain systems level
Resource allocation
Time

Figure 1. Hypothesized reallocation of neural resources of the salience (emotion) and cognitive control network upon trauma exposure in the healthy and PTSD brain (adapted from 16). We propose to investigate the abnormalities in neural network function at baseline (scenario 1), upon trauma exposure (scenario 2), and following recovery (scenario 3).

Although translational value has to be warranted, animal studies provide the clear advantage of more invasive sampling techniques and methods for intervention, while allowing for tightly controlled prospective studies. Here, I propose to use a previously validated mouse model for PTSD-induction (17-19, see also Ru-DEC 2014-175 & Ru-DEC 2014-243), in which mice are [redacted] exposed to a severe stressor (i.e., intense electric footshock) [redacted] This protocol, [redacted] has been shown to reliably induce PTSD-like symptomatology - i.e., hypervigilance, insomnia, compulsivity, and impaired attention and risk assessment (2) – [redacted] Here, we aim to elucidate the microscopic neuronal circuit function differentiating the PTSD-vulnerable from [redacted] brain to enhance the understanding of PTSD etiology to improve its detection and treatment. We will do so in three steps. First, we will identify and characterize the neuronal subpopulations that show differential activity or plasticity in PTSD-[redacted] mice. Next, we will identify the neuronal circuits they are part of (and by which they establish their effects). Last, we will manipulate the activity (or plasticity) of these neuronal circuits to [redacted] and deliver causal evidence for the observed network changes and the PTSD-like phenotype. As it is important to determine when the neuronal network changes [redacted] arise, we will address each of these steps of identifying aberrant neuronal network function at baseline (research question #1), during trauma exposure (research question #2), or following trauma recovery (research question #3).

NB. As will become evident in the rest of the proposal, we will only use male mice for the proposed studies. We acknowledge the sex difference in sensitivity to PTSD, and think future dedicated studies should most certainly address this. Here we however chose to restrict ourselves to the sex with the most robust and stable stress response, in which the proposed PTSD model has been established (this is not the case for females). Since stress sensitivity is dependent on the hormonal cycle of females (25,26), as is the brain response to stress (25), testing of females would require three times the amount of animals and close monitoring of their estrous cycle.

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3. Kessler RC, Chiu WT, Demler O, Walters EE (2005). Prevalence, severity, and comorbidity of twelve-month DSM-IV disorders in the National Comorbidity Survey Replication (NCS-R). *Archives of General Psychiatry* 62(6): 617-627.
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9. Beckmann CF, DeLuca M, Devlin JT, Smith SM (2005). Investigations into resting-state connectivity using independent component analysis. *Philos Trans R Soc Lond B Biol Sci* 360: 1001-1013.
10. Whitfield-Gabrieli S, Ford JM (2012). Default mode network activity and connectivity in psychopathology. *Annu Rev Clin Psychol* 8: 49-76.
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16. [redacted]
17. [redacted]
18. [redacted]

19. [redacted]
20. Rau V, DeCola JP, Fanselow MS (2005). Stress-induced enhancement of fear learning: an animal model of posttraumatic stress disorder. *Neurosci Biobehav Rev* 29: 1207–1223.
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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 *overall objective* of this project is to elucidate aberrant neuronal circuit function differentiating the PTSD-[redacted] brain to enhance the understanding of PTSD etiology.

The following 3 *research questions* will be addressed to meet our objective:
1. Which neuronal circuit function *<u>at baseline</u>* is predictive of PTSD-development?
2. Which neuronal circuit *<u>response to trauma exposure</u>* predicts PTSD-development?
3. Which neuronal circuit function is associated with PTSD-pathology and resiliency *<u>following trauma recovery</u>*?

Feasibility
This research project [redacted] We have the experience and facilities in-house to perform the required studies. Researchers involved in this proposal have extensive experience with the proposed PTSD model, as well as the use of the proposed techniques (qPCR, immunohistochemistry, neuronal tracing, and optogenetics) for the experiments. Moreover, in collaboration with others, the researchers have already performed (successful) preliminary tests in the proposed transgenic mouse lines. The use of an available light-sheet microscope will further facilitate the analyses of fluorescent labeling.

3.3 Relevance

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

Estimates are that up to 90% of all people will be exposed to a severe traumatic event during their life time, of which a substantial part (15-20%) ultimately develops PTSD (1). This makes PTSD the fourth most common psychiatric diagnosis, which annually affects 7.7 million adults in Europe only (2). Patients typically experience severe re-experiencing symptoms of the traumatic event, which can manifest themselves as flashbacks, nightmares, or frightening thoughts. Moreover, they often show emotionally numbing, feel strong guilt, depression, or worry and loose interest in activities that were enjoyable in the past. They are also characterized by a state of hyperarousal; they are easily startled, feel tense "on edge", often have difficulty sleeping, and have angry outbursts. Thereby, patients feel stressed or tensed continuously, and are often unable to do simple daily tasks, such as sleeping, eating, or concentrating.
Besides severely affecting one's quality of life, PTSD, and anxiety disorders in general, form a major financial burden on society. The annual cost to society of anxiety disorders is estimated to be significantly over €9 billion in Europe only, often due to misdiagnosis and under treatment (2). This includes psychiatric and non-psychiatric medical treatment costs, indirect workplace costs, mortality costs, and prescription drug costs. Up to date, there is no good medication to treat PTSD and other fear-related disorders. Although there are several existing pharmacological treatments, all of these rely on empirically derived approaches. The first-line medication approach for all anxiety disorders includes the antidepressant and anxiolytic classes of selective and non-selective serotonin and other monoamine reuptake inhibitors. It is clear these are not specific in their actions, they can have difficult side effects, and they are only effective in some cases. The second most-common class of agents to treat these disorders are the benzodiazepines, which act through enhancement of GABA activity, which have been shown successful in diminishing fear responses, but have the same limitations as the monoaminergic anxiolytics, in addition to having abuse and tolerance potential. Increasing our understanding of the neurobiological basis of PTSD can provide us with new leads for drug treatment.
Therefore, it is of major importance to society that PTSD is identified in an earlier phase, and that treatment efficacy improves. This goal can only be achieved when our understanding of the underlying neuronal basis of the disorder is increased. The experiments described in this protocol are expected to significantly contribute to exactly this. Answering research question #1 will enable us to identify vulnerability factors that characterize the brain at risk for PTSD even prior to trauma exposure. Answering experimental question #2 will inform us on the mechanistic underpinnings of both the adequate and inadequate response to trauma exposure, and answering experimental question #3 will inform us on the pathology indicative of the PTSD-like brain. If these findings can be translated to suitable biomarkers in humans (e.g., by taking blood samples for stress hormone (corticosterone) measurement, fMRI recordings to assess regional brain activity or plasticity, or genetic analyses indicative of protein levels), they could contribute to PTSD prevention and early diagnosis of those at risk, and new potential targets for intervention/treatment, creating new leads for the development of PTSD-specific medication. Furthermore, the obtained insights could contribute to a better understanding of stress-related and anxiety disorders in general, such as major depression and general anxiety disorder.

References

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2. Olesena J, Gustavsson A, Svensson M, Wittchene HU, Jönsson B (2012). The economic cost of brain disorders in Europe. *Eur J Neurology* 19: 155-162.

3.4 Research Strategy

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

The *overall objective* of this project is to elucidate aberrant neuronal circuit function differentiating the PTSD-[redacted] brain to enhance the understanding of PTSD etiology to improve its detection and treatment.

The following **3 related, though conceptually independent,** research questions will be addressed to meet our objective:
1) Which neuronal circuit function *at baseline* is predictive of PTSD-development?
2) Which neuronal circuit *response to trauma exposure* predicts PTSD-development?
3) Which neuronal circuit function is associated with PTSD-pathology and resiliency *following trauma recovery*?

Since it is critical in this proposal that the mice are behaviorally categorized as [redacted] of PTSD-like at the end of the paradigm (and thus survive until this final stage), this project requires tools to look into neuronal activity and plasticity retrospectively. That is, we need a technique to label neuronal activity and plasticity at a certain stage (at baseline, during trauma, following recovery) and link this to behavioral outcome of the PTSD-induction procedure in the end. Recently, two transgenic mouse strains have been developed that enable us to do so; [redacted]. Here, we will use these mice to study the PTSD-[redacted] brain at different stages throughout PTSD-development.

Since we will start by assessing neuronal activation during periods of relative rest in case of research question #1 & #3, compared to a period of exposure to a defined stimulus in case of research question #2, tackling these research questions asks for a somewhat different approach.

For research questions #1 & #3 our approach is as follows:
A) First, we will determine the neuronal activation patterns dissociating the PTSD-[redacted] brain using indelible labeling of neuronal activation (with a fluorescent marker) in two specific transgenic mouse lines at either baseline (#1) or following recovery (#3). Next, once the neuronal populations displaying aberrant activation associated with PTSD-outcome have been identified, we will characterize them in terms of neurobiological make up using **quantative PCR (qPCR), followed up by *in situ* hybridization and** immunohistochemistry **experiments to determine the specificity of the obtained results**.
B) Then, we will perform neuronal tracing studies on the identified neuronal subpopulation to determine their projection sites and identify the neural circuit affected.
C) Lastly, we will manipulate the activity and connectivity of this neuronal circuit to prevent (# 1) or treat (# 3) the PTSD phenotype respectively.
For research question #2 our approach will be slightly different:
A1) First, we start by identifying which brain regions actively respond to the entire PTSD-induction procedure ([redacted]) and test for any differences between PTSD-[redacted] animals. To be able to identify stress responsive brain regions at this stage, we will also include a control group (for each transgenic strain), which will not be exposed to the PTSD-induction procedure, but will be exposed to all other

treatments (e.g., [redacted]). This first experiment will mainly serve as a proof of principle (testing whether we can observe differential labeling (activity/plasticity) patterns in stress-related brain regions [redacted]), and will provide us with regions-of-interest for further investigation.
A2) Next, we will try to narrow down the time window of aberrant neuronal activation by looking into [redacted]-induced neuronal activation specifically ([redacted]), providing us with more detailed (and sensitive) information on the required activation patterns for PTSD-development. [redacted] Again, a control group will be included here as well. The neuronal populations will be compared and characterized in terms of neurobiological make up using **quantative PCR (qPCR), followed up by *in situ* hybridization and** immunohistochemistry **experiments to determine the specificity of the obtained results**.
B) Then, we will perform neuronal tracing studies on the most promising neuronal population (of A1 and A2) to determine its projection sites and identify the neural circuits affected.
C) Finally, we will manipulate the activity and connectivity of this neuronal circuit to intervene with PTSD-development.

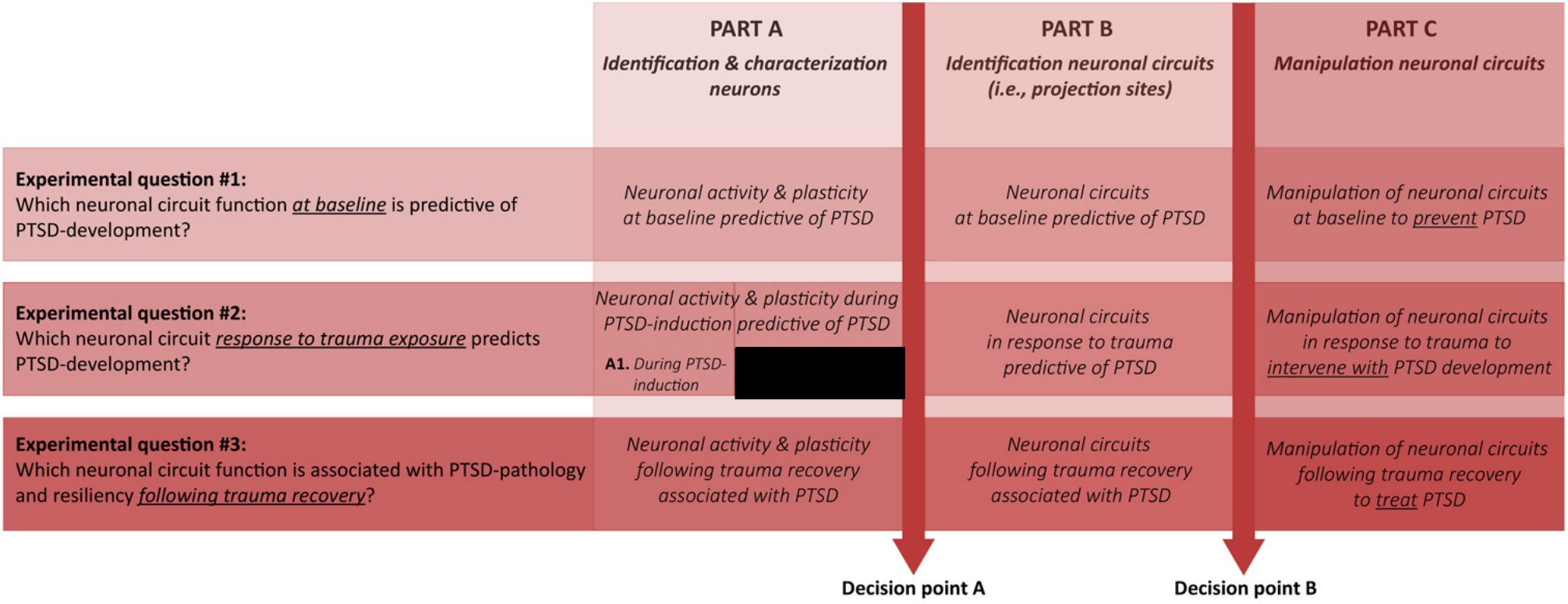

Figure 2. Experimental outline of the project proposal. The three experimental questions will be addressed in separate, independent experiments (1-3), of which each consists of three parts (A-C) that are dependent on each other. Therefore, two decision points will be implemented (A & B), at which is determined whether continuation of the experiment to parts B and C should be pursued (see section 3.4.3. for more details).

References

1. [redacted]
2. [redacted]

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.

As explained in 3.4.1, the characterization of aberrant neuronal signalling associated with PTSD will occur at three different stages (i.e., baseline, in response to trauma, and after recovery) and will be investigated in several subsequent steps **(parts A-C)**. First, the affected neuronal population is identified and will be characterized in terms of neurobiological makeup. Next, its projection sites will be determined. Lastly, the corresponding neuronal circuit is manipulated [redacted], to deliver causative evidence.

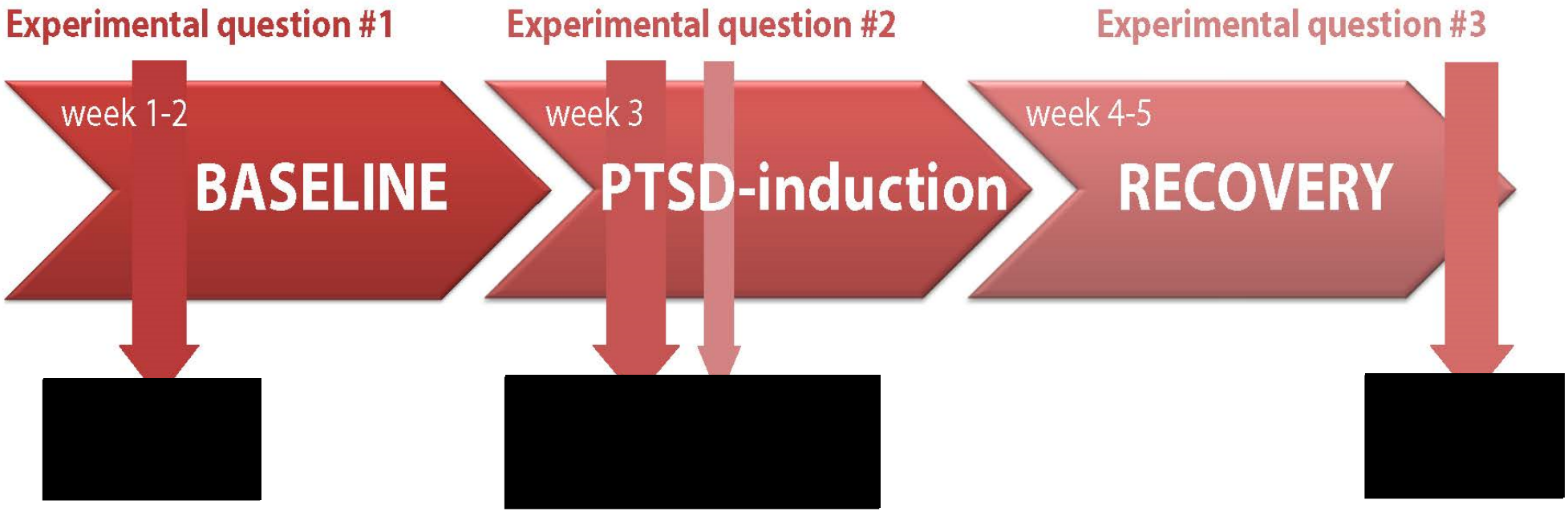

Figure 3. Experimental time line of the proposed experiments. [redacted] at baseline will occur for experimental question #1, just prior to PTSD-induction for research question #2, and following trauma recovery for research question #3. The labeling of neuronal activity/plasticity during these time periods will be compared [redacted] animals and thereby allow for the identification and characterization of aberrant neuronal circuit function associated with PTSD. [redacted]

PTSD-induction protocol

For all of these studies, mice will be exposed to a well-established mouse PTSD model ([redacted]) to induce a PTSD-like phenotype [redacted]. Briefly, animals are exposed to a trauma (electric foot shock) [redacted]. Animals get a week to recover and are subsequently phenotyped on typical PTSD-behaviors ([redacted]), [redacted] A detailed description of individual tests is provided in the Animal Procedures.

Part A.
To identify aberrant neuronal activation and plasticity in PTSD animals, two transgenic mouse lines will be used, Mice are subjected to the PTSD-induction protocol and either at baseline (question #1), just prior to PTSD-induction (question #2, A1), or after recovery once the pathology has been established (question #3), to indelibly label their neuronal activity/plasticity patterns in these corresponding periods. At the end of the protocol, animals will be sacrificed and neuronal labeling in PTSD brains will be compared. Moreover, to answer question #2, we will narrow down the time window of aberrant neuronal activation and plasticity by looking into trauma -induced neuronal activation and plasticity separately, providing us with more detailed (and sensitive) information on the required activation and plasticity patterns for PTSD-development. To characterize the neurobiological makeup of the activated/plastic neuronal populations (differentiating the PTSD-susceptible from brain) we will **first sort (i.e., isolate) the fluorescently labeled cells using Fluorescence-Activated Cell Sorting (FACS) and perform quantitative PCR on the cells to identify activity/plasticity- and stress-related genes with 1) high expression levels in the identified neuronal subpopulation (which could potentially serve as cell marker in Part B), or 2) altered expression levels , which is informative on the underlying cause of altered activity/plasticity in these cells. These experiments will be followed-up by** immunohistochemistry and *in situ* hybridization experiments on the brain slices acquired from the **first batch of** animals**, to determine the specificity of the qPCR findings (i.e., whether the expression of certain genes is restricted to the identified cell population) and inform us on the potential of certain cell markers for Part B**.

Part B.
Next, we will identify the projection sites of the neurons identified in Part A (and thus the neuronal circuit affected) by intracranial injection of Cre-dependent viral vectors (expressing a fluorescent label) in the brain regions of interest and subsequent analyses of its expression sites. Depending on the neuronal subpopulation identified in Part A (decision point A), this injection will either happen in mice prior to the PTSD-induction protocol and , or in specific Cre-lines for the neuronal subpopulation identified (as further specified in the Animal Procedures).

Part C.
To manipulate the neuronal circuit we will make use of optogenetics, a technique in which the activity of genetically modified neuronal populations can be manipulated by light. In these experiments, the intracranial injection of a viral vector expressing the opsin might be required, as well as the implantation of optic fibers for local light delivery. Depending on the neuronal circuit identified in Part B, several scenarios for optogenetic manipulation are possible (decision point B), which are described in detail in the Animal Procedures.

References
1.
2.

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

As described in 3.4.1. (Research Strategy), the approach of this project is to perform the **experiments necessary to answer our 3 (related, but independent) research questions** in a sequential manner.
- Firstly, a screening of neuronal activation/plasticity patterns will be performed, comparing the PTSD-[redacted] brain at several stages throughout PTSD-development, to identify the most pronounced differences in neuronal activity/plasticity (part A), followed by a characterization of this neuronal cell population in terms of gene-expression patterns and neurobiological makeup.*
- Secondly, this identification of affected neurons will be followed up by a more in-depth characterization of the neuronal circuits they are part of (Part B).**
- Finally, these neuronal circuits in the PTSD-[redacted] brain are manipulated to [redacted], and thereby provide evidence for a causal relationship between the circuit and PTSD-[redacted] (Part C).
Thereby, this project will produce output parameters covering a wide spectrum from the macroscopic systems level to the microscopic molecular level; ranging from behavioral parameters (anxiety levels, exploration, etc.), to neuronal circuits, activation patterns and neurobiological makeup. These data will be combined and compared across experiments, to - in the end - sketch a coherent picture of the neuronal activation dissociating the PTSD-[redacted] brain. The identification of PTSD risk factors (at all these levels), predicting PTSD-development even prior to trauma exposure (question #1), will be one milestone. The identification of immediate response factors to trauma exposure predicting later PTSD-development (question #2), will be a second milestone. The identification of biomarkers related to PTSD pathology (question #3) will be a third milestone.

*: Decision point A: If in the first part of the proposal (Part A) no clear neuronal target (i.e., neuronal subpopulation) can be identified for one of the questions #1-#3 (i.e., clearly abnormal neuronal actvity/plasticity of a neuronal subpopulation at baseline (#1), in response to trauma exposure (#2), and following recovery (#3)), the second (Part B) and third steps (Part C) of the proposal will not be pursued for this question. Practically, this means that if we do not observe significant differences in the amount of labeled neurons (indicating their history of activity/plasticity) in any brain region for the PTSD-[redacted] animals, part B and C will not be pursued using the same protocol. If such significant differences are observed, we will focus on the brain region with the largest difference (i.e., effect size) and most homogeneous cell population identified.
**: If in the second part of the proposal (Part B) no clear neuronal target (i.e., neuronal circuit) can be identified for one of the questions #1-#3 (i.e., clearly abnormal neuronal actvity/plasticity of a neuronal circuit at baseline (#1), in response to trauma exposure (#2), and following recovery (#3)), the third step (Part C) of the proposal will not be pursued for this question. Practically, this means if we cannot identify significant projections of the neuronal subpopulation identified in Part A, we will refrain from attempting to manipulate this circuit in Part C. If sparse projections are observed (but consistent among animals) we will manipulate the activity/plasticity of the cells (from Part A) themselves in Part C, instead of performing photostimulation of their projection sites.

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	A. Identification of PTSD-associated neuronal activation and plasticity
2	B. Characterization of PTSD-associated neuronal circuits
3	C. Manipulation of PTSD-associated neuronal circuits

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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 1	Type of animal procedure A. Identification of PTSD-associated neuronal activation and plasticity

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.

General design:
Our approach is to implement a PTSD-induction protocol, existing of a trauma (electric shock) followed by [redacted] to induce PTSD-like behavior [redacted] animals. A week after PTSD-induction, animals will be tested in a set of behavioral tests (i.e., [redacted]) assessing PTSD-symptomatology, and their neuroendocrine function will be tested (corticosterone response to restraint [redacted] In order to obtain insight into the neuronal activation and plasticity patterns associated with PTSD, neuronal activity and plasticity will either be labeled at 1) baseline, 2) in response to PTSD-induction (i.e., trauma [redacted] exposure), or 3) following recovery (once the pathology has been established), and PTSD- [redacted] animals will be compared. The labeling of neuronal activation and plasticity will occur by making use of two transgenic mouse lines, [redacted], each targeting a different subset of neurons; those displaying increased neuronal firing (i.e. activity) vs. those displaying increased neuronal plasticity, respectively. [redacted] Three weeks after the end of the experimental protocol, animals will be sacrificed**. Two groups of mice (in parallel) will be used for each experiment, of which one will be sacrificed by decapitation to perform gene-expression analyses on the labeled cells using FACS and qPCR to identify activity/plasticity-related genes and stress-related genes with either high overall expression levels in the fluorescently labeled cells (which could potentially indicate their potential as neuronal marker for Part B of the proposal), or altered expression levels in the labeled cells of PTSD- [redacted] animals; informing us on the underlying mechanisms of the observed differences in neuronal plasticity/activity in these groups. The other group will be sacrificed by** perfusion fixation **to** analyze **their brain** for expression of the fluorescent marker**, and perform *in situ* hybridization and immunohistochemistry experiments to characterize their neurobiological make up and identify potential neuronal markers for Part B of the proposal**.

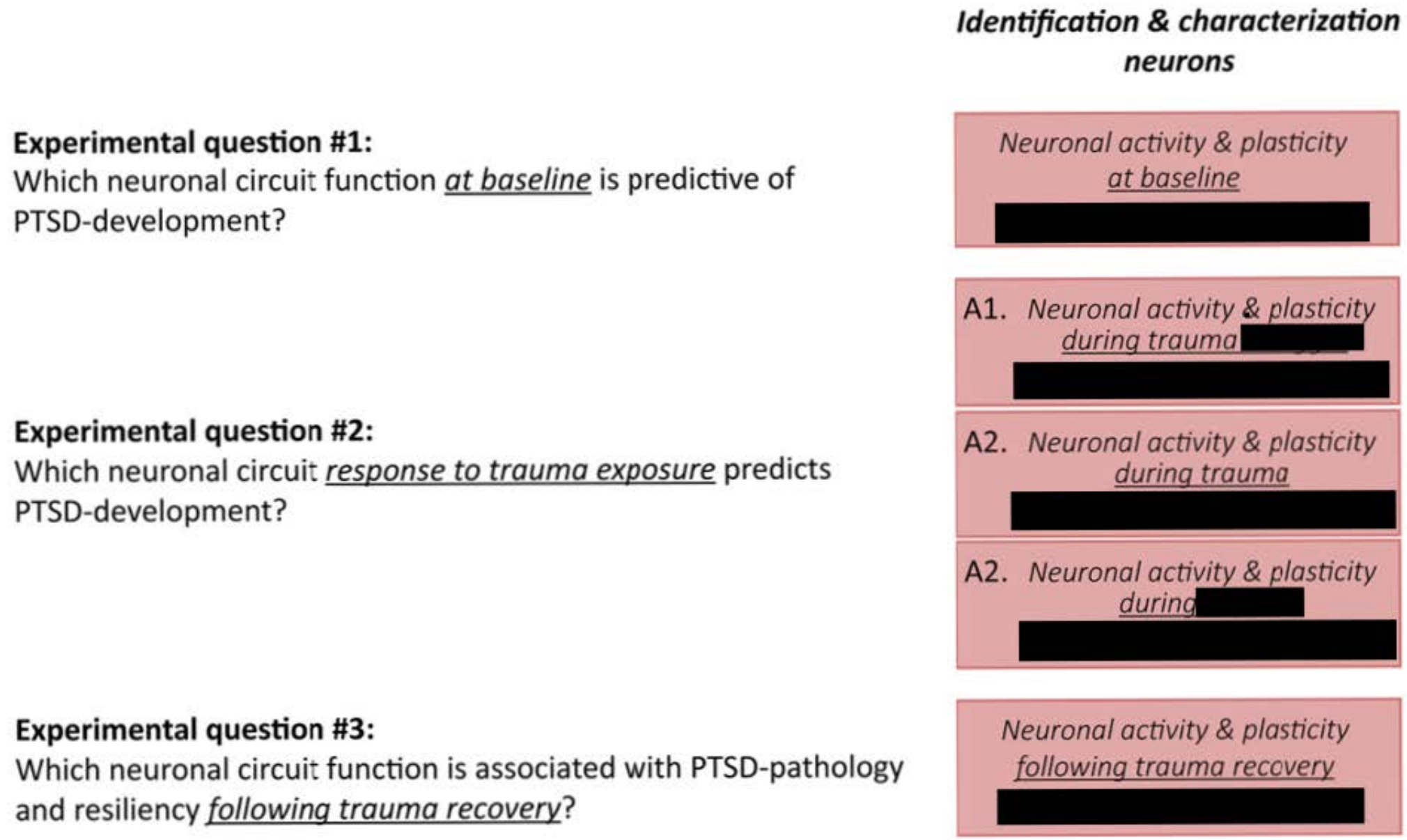

Figure 4. Experimental design of Part A.

Primary outcome parameters:
- Behavioral phenotype; [redacted]
- Neuroendocrine function; [redacted]
- Pattern of neuronal activation and plasticity at baseline (question #1; [redacted]) & **gene expression pattern and** neurobiological makeup of the identified cells
- Pattern of neuronal activation and plasticity in response to trauma[redacted] exposure (question #2; [redacted]) & **gene expression pattern and** neurobiological makeup of the identified cells
- Pattern of neuronal activation and plasticity following recovery (question #3; [redacted]) & **gene expression pattern and** neurobiological makeup of the identified cells

Justification:
The set of behavioral output measures is critical [redacted]. Moreover, since neuroendocrine abnormalities are associated with PTSD, we include these measures as well to try to associate them with aberrant neuronal activation and/or plasticity in PTSD. The differences in neuronal activation and/or plasticity between [redacted] and PTSD-like animals at baseline will inform us about neuronal activation constituting a risk factor for PTSD-development. The differences in neuronal activation and/or plasticity between [redacted] and PTSD-like animals in response to trauma/[redacted] exposure will inform us about neuronal activation constituting an immediate marker of PTSD-risk in reaction to trauma exposure. The differences in neuronal activation and/or plasticity between [redacted] and PTSD-like animals after recovery informs us about neuronal activation reflecting PTSD-pathology, and is therefore a useful target for treatment.

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

I. Basal anxiety

Open Field Test.

To assess basal anxiety, mice will be tested in the open field test. This test is based on the animals' natural conflict between exploration of and the aversion against open, bright areas. The open field apparatus consists of a white Plexiglas box (50 x 50 x 40 cm) lightened with 120 lux. Each mouse will be placed in the corner of the apparatus to initiate a 10 min test session. Time spent in the center (the inner 25 x 25 cm), distance traveled in the center, number of visits to the center, and total distance traveled will be quantified using a camera mounted above the apparatus and analyzed by Ethovision software (Noldus, Wageningen, Netherlands).

Elevated Plus Maze.

As a second test for basal anxiety, the elevated plus maze will be used, which is also makes use of the rodents' aversion of open spaces. The elevated plus maze comprises a central part (5 x 5 cm), two opposing open arms (30.5 x 5 cm), and two opposing Plexiglas closed arms (30.5 x 5 x 15 cm), elevated at a height of 53.5 cm and the open arms are illuminated with 6-9 lux. Mice are placed in one of the closed arms facing the center to initiate a 5 min test session. Time spent in the open arms, distance traveled in the open arms, and number of visits to the open arms will be quantified using a camera mounted above the apparatus and analyzed by Ethovision software (Noldus, Wageningen, Netherlands).

II. PTSD-induction

For these studies, male, adult mice will be exposed to a well-established mouse PTSD model ([redacted]) to induce a PTSD-like phenotype [redacted] animals. The model begins on day [redacted], in which mice receive 14 shocks of 1 mA, 1 s in duration over 85 min at variable intervals, representing the "trauma". On day [redacted]. Shocks will be given in a fear-conditioning apparatus. [redacted] sloping roof placed on the metal grid floor, [redacted]

III. Behavioral tests for PTSD identification (phenotyping)

Mice are tested in five behavioral tests to determine whether they developed PTSD, each assessing different aspects of PTSD-symptomatology: [redacted]

One of the features of PTSD tested for is the impairment in risk assessment. In PTSD patients this dysfunction often manifests itself as paranoia (4) and risky behavior demonstrated by high incidences of violence, drug abuse, or suicide (5,6).The PTSD-like mice also show risk assessment patterns consistent with an immediate or imminent danger in the face of a predator and not to an uncertain potential for risk. Mice normally engage in oriented information-gathering scanning from place of concealment and increases in stretch attend posture (7,8) in the absence of a predator, but in the presence of a predator this activity is reduced in favor of quick flight. Increased risk assessment is also associated with reduced anxiety (9).
We will measure risk assessment using the dark/light transfer test (1). The test apparatus consists of a box divided by a partition into two environments: a dark covered compartment (15 x 20 x 25 cm) and a brightly illuminated (1000–1100 lux) light compartment (30 x 25 x 25 cm). The compartments are connected by a small passage in the bottom center of the partition. The mice are placed in the dark compartment to initiate a 5 minutes test session. Time spent in the light zone, number of visits to the light zone and the latency entering the light zone will be quantified using a camera mounted above the apparatus and analyzed by ([redacted]). An additional arena of 3 cm lengthwise by 6 cm width-wise will be programmed into the software tracking measurements surrounding the opening of the light area. Time spent in the risk assessment area and the number of visits to the risk assessment area are measured. Percentage risk assessment time will be calculated as the amount of time spent in the risk assessment arena as a percentage of total time spent in the light area outside of the risk assessment zone. [redacted]

Latency to peak startle amplitude and pre-pulse inhibition.
Exaggerated startle is one of the DSMIV criteria for PTSD (10), reflecting hyperarousal in patients. Moreover, impaired pre-pulse inhibition, a measure of sensorimotor gating but also a test that requires attentional processes, has been reported for PTSD (11). To assess the animals' (latency to) peak startle and the amount of pre-pulse inhibition we here use an acoustic startle protocol. The proposed protocol is similar to those reported before (1,12). Briefly, mice are placed in a small Plexiglas cage on top of a vibration-sensitive platform in a sound-attenuated, ventilated chamber. A high-precision sensor, integrated into the measuring platform, detects movement. Two high-frequency loudspeakers inside the chamber produce all the audio stimuli. The acoustic startle response (ASR) session begins with 5 min acclimation to white background noise (70 dB) maintained through

the whole session. Thirty-two startle stimuli (120 dB, 40 ms in duration with a randomly varying ITI of 12–30 s) are presented interspersed with an additional 40 startle stimuli randomly preceded by 40 ms prepulses of either 74 dB, 78 dB, or 82 dB. Maximal ASR and latency to peak startle amplitude are measured both in response to individually presented startle stimuli and in response to startle stimuli preceded by pre-pulses. Percentage pre-pulse inhibition (PPI) will be calculated as the percent difference between the maximal ASR (max G) to startle stimuli preceded by pre-pulses compared to that without. [redacted]

Marble burying.
Marble burying will be assessed to measure hypervigilance and overall anxiety in the animals (13). Mice are placed in a compartment illuminated by 10 lux with dimensions (30 × 27 × 26 cm) containing 5 cm autoclaved bedding with 20 marbles centrally arranged 4 by 5. Mice are then filmed for 25 min. Videos are scored by counting the number of unburied marbles every 5 minutes until the end of the test (14). [redacted]

Homecage locomotion.
Homecage locomotion is assessed using the observation (Phenotyper) cages. Mice are housed individually for 72 h, in which the first 24 h are considered habituation to the individual housing conditions. Measurements of general locomotion consist of two light and two dark cycles in the last 48 h collected at 10 min intervals (1). [redacted] This test models the sleeping problems PTSD patients suffer from (10). Most patients will reach a clinical setting initially due to insomnia or disturbing nightmares, which to date have no specific cure.

Evaluation of [redacted]

IV. Assessment of neuroendocrine function
Based on **the suggested role of the stress hormone corticosterone in stress recovery (15,16) and** the observation of neuroendocrine abnormalities in PTSD-patients, be it either in basal state or following a challenge (17,18), we will also monitor neuroendocrine function [redacted] mice over the course of PTSD-development, and later correlate these measures with neuronal activation. This will inform us on the potential causal relationship between neuroendocrine signaling and brain function and the supposed potential of corticosterone administration as treatment for PTSD (19,20). Therefore, corticosterone levels will be assessed by tail bleed (10 µL) 12 times over the course of the experiment.

- Two repetitions of basal corticosterone measurements in the morning (at the circadian peak) and evening (at the circadian trough); both at the start and at the end of the experiment (total = 8 measurements)

- Stress response corticosterone levels will be assessed in response to trauma and trigger (total = 2 measurements)

- Stress response corticosterone levels will be assessed in response to restraint stress; both at the start and at the end of the experiment, once pathology has been established (total = 8 measurements)

V. Restraint stress

To measure the corticosterone stress response and subsequent recovery, animals will be exposed to 25 min restraint stress in plastic restrainers. Plasma will be extracted from blood samples (10 µL) that are collected by tail bleed at four time points: under basal conditions, at 25 min (i.e., immediately when removed from the restrainer), 75 min, and 120 min following stress initiation. This exact protocol has been used before to show abnormal corticosterone responding to stress in PTSD-like animals (1), and correlational analyses with the brain findings will inform us on the neural basis of these changes.

VI. [redacted]

In order to label the neurons displaying activity or plasticity over a certain time bin (baseline, trauma/[redacted] exposure, after recovery), mice will be [redacted] at different time points in the experimental design.Moreover, to answer question #2, we will narrow down the time window of aberrant neuronal activation and plasticity by looking into trauma & [redacted]-induced neuronal activation and plasticity separately, providing us with more detailed (and sensitive) information on the required activation and plasticity patterns for PTSD-development. [redacted] is absolutely critical for [redacted] Thereby we will get more detailed temporal-dynamic information and less background signal about adaptive and maladaptive responding to the traumatic experience. Such temporal precision is not required for the labeling of basal neuronal activity and plasticity (as targeted for questions #1 and #3), making that [redacted] will be sufficient in those cases.

VII. Sacrifice

Group 1. For histological read-out of neuronal activity/plasticity **in terms of fluorescent labeling, as well as later *in situ* hybridization and immunohistochemistry experiments,** animals will be sacrificed by an overdose of anesthesia followed by transcardial perfusion with saline and fixative.

Group 2. For the quantification of gene expression in the identified neurons (displaying aberrant activity/plasticity associated with a PTSD-like phenotype), animals will be sacrificed using rapid decapitation (with no anesthesia).

The total duration of the experiment will be 3 months maximum.

VIII. Identification and characterization of neuronal populations

Group 1. Following the transcardial perfusion, brains will be extracted, and brain slices will be prepared. One set of brain slices will be used for the identification of neuronal populations (and brain regions) displaying aberrant activity/plasticity in PTSD-[redacted] animals (done by the comparison of the counted number of labeled cells). The other sets of brain slices will be used for immunohistochemistry and *in situ* hybridization experiments to characterize the cells in terms of excitatory or inhibitory nature, and their expression of activity-, plasticity- and stress-related proteins, as well as activity-, plasticity-, and stress-related gene transcription.

Group 2. Following decapitation, brains will be extracted and immediately frozen on dry ice. Punches of relevant brain regions (as identified in group 1) will be obtained and prepared for FACS (Fluorescence-Activated Cell Sorting). Following the isolation of the fluorescently labeled cells, qPCR experiments will be performed to analyse the expression patterns of stress-related and activity/plasticity-related genes.

References

1. [redacted]
2. [redacted].
3. [redacted]
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20. Zohar J, Yahalom H, Kozlovsky N, Cwikel-Hamzany S, Matar MA, Kaplan Z, Yehuda R, Cohen H (2011). High dose hydrocortisone immediately after trauma may alter the trajectory of PTSD: interplay between clinical and animal studies. *Eur Neuropsychopharmacol* 21(11): 796-809.

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

Since Part A involves a brain wide scan for potential brain regions and neuronal subpopulations of interest, we are rather conservative in the numbers of animals included in this section, to ensure proper statistical power to identify targets. [redacted]. Previous studies implementing this strategy (1-3), have already successfully identified neural differences between these groups. Moreover, the all-or-nothing strategy mimics the human situation, in which patients are either diagnosed with PTSD (meeting multiple criteria of stressor exposure, intrusion symptoms, avoidance, alterations in arousal and reactivity, and negative alterations in cognition and mood) or not (4). For this initial screen, we estimate to need 12 mice per group. We will calculate the precise group sizes per experiment using a power analysis, based on data collected so far by us and others. As only 25% of all trauma-exposed animals is expected to either display a PTSD-like phenotype [redacted], we have to expose 48 mice per group to end up with these group sizes.

Once these first analyses have identified interesting targets, we will explore correlational analyses between these outcome measures and PTSD-scores of all animals, to test whether correlational analyses are suitable and sufficient for the experiments described in Part B and C of this proposal.

The following groups, as introduced in the project proposal, will be assessed:

[redacted]
1. PTSD-induction [redacted] at baseline (question #1)
2. PTSD-induction [redacted] at trauma [redacted] (question #2a1)
3. PTSD-induction [redacted] at trauma (question #2a2)
4. PTSD-induction [redacted] (question #2a2)
5. PTSD-induction [redacted] at end (question #3)

= 5x 48 mice, is 240 mice.

Furthermore, to be able to specifically identify neuronal activity/plasticity specifically related to the trauma/ exposure (instead of to e.g., stress or novelty induced stress) also 12 control animals will be included for groups 2-4, which are not exposed to PTSD-induction, but do receive the injection. Moreover, the inclusion of these control animals will inform us about the adaptive (adequate) response to the trauma in the animals. This all adds up to mice **per experiment** in total.

Arc-CreERT2
6. PTSD-induction at baseline (question #1)
7. PTSD-induction at trauma (question #2a1)
8. PTSD-induction at trauma (question #2a2)
9. PTSD-induction (question #2a2)
10. PTSD-induction at end (question #3)

= 5x 48 mice, is 240 mice.
to be able to specifically identify neuronal activity/plasticity specifically related to the trauma exposure (instead of to e.g., stress or novelty induced stress) also 12 control animals will be included for groups 7-9, which are not exposed to PTSD-induction, but do receive the injection . Moreover, the inclusion of these control animals will inform us about the adaptive (adequate) response to the trauma in the animals. This all adds up to mice **per experiment** in total.

Since we need both perfused brain tissue (to initially identify the brain regions of interest with neurons displaying aberrant activity/plasticity by fluorescent cell counting, and perform later *in situ* hybridization and immunohistochemistry experiments), and fresh brain tissue (to analyze the gene-expression patterns associated with this altered function (characterizing the neurons)), we will need two groups for each experiment, which are sacrificed by perfusion fixation and decapitation, respectively.

Thus, we need to double the number of animals; resulting in a total of 1104 animals required for these experiments.

N.B. Throughout all procedures data dropout and loss of animals will be minimized by careful execution of the experiments and close monitoring animal welfare.

References

1.
2.
3.

4. American Psychiatric Association (1994). *Diagnostic and statistical manual of mental disorders (DSM IV)* (Washington, DC, American Psychiatric).

B. The animals

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

The mouse is the lowest animal species in which we are able to model human psychiatric disorders. Because of the complexity of the brain and symptomatology to diagnose these disorders, it is impossible to use lower-order animals or organs. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and type of trauma exposure, and all pre-, peri-, and post-trauma factors potentially influencing an eventual disease outcome. This is not possible in humans.
Furthermore, the proposed PTSD-protocol has been validated in mice. Here, we propose to use mice from two specific transgenic mouse lines; [redacted] Both transgenic lines label a distinct set of neurons and have their own characteristics in terms of selectivity (background labeling) and sensitivity ([redacted]), and therefore of added value to each other. Moreover, PTSD in human patients has been linked to both abnormal neural activity (2,3) and connectivity - reflecting plasticity - (4,5), making it necessary to target both processes independently.

References:

1) [redacted].
2) Bremner JD (2002). Neuroimaging studies in post-traumatic stress disorder. *Curr Psychiatry Rep* 4: 254-263.
3) Shin LM, Rauch SL, Pitman RK (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci* 1071: 67-79.
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Species	Origin	Maximum number of animals	Life stage
Mouse	[redacted]	1104	adult (>8 weeks old)

C. Re-use

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 mouse is the lowest animal species in which we are able to model human psychiatric disorders. Because of the complexity of the brain and symptomatology to diagnose these disorders, it is impossible to use lower-order animals or organs. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and type of trauma exposure, and all pre-, peri-, and post-trauma factors potentially influencing an eventual disease outcome. This is not possible in humans.

Reduction

The requested amount of animals (based on a group size of n = 12) is needed for statistical reliable conclusions and is the minimal group size one can work with. As only a subset of the total amount of trauma-exposed animals will develop PTSD (25%) and [redacted] the total group size has to be (4x) larger. Following the identification of neuronal subpopulations of interest (in which differences betweeon PTSD-like [redacted] animals are observed), we will explore correlational analyses between these outcome measures and the PTSD-score in all animals to test whether correlational analyses are also a useful tool to explore differences in PTSD-symptomatology. If this is the case, we will refer to correlaitonal analyses for the experiments described in Part B and C of this proposal.

Refinement

The experiments will be carried out with the least discomfort possible. However, exposure to electrical shocks (and the discomfort caused by them) is critical to the induction of PTSD and therefore unavoidable.

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

Although the PTSD-induction procedure will be aversive to the animals, no physical adverse effects are expected. Animals will be monitored closely for signs of discomfort and checked daily to be able to detect Humane End Point conditions. Handling of the animals will be minimized to reduce human intervention that may cause stress for the animals. Also, habituation to the housing at a reverse day/night rhythm will be applied. Perfusion will take place under deep anesthesia to minimize adverse effects.

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.

N/A

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?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

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

[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?

[X] No > Justify why pain relieving methods will not be used.

The electric footshocks required for PTSD-induction will cause discomfort in the animals and will most likely be experienced as slightly painful. However, this procedure (and the discomfort caused by it) is critical to our manipulation; no pain relief can be used.

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

I. Other aspects compromising the welfare of the animals

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

The mice will experience severe discomfort during the PTSD-induction procedure, moderate stress during the restraint stress session, mild discomfort due to the [redacted] and tail blood collection, and very mild psychological discomfort in the behavioral tests assessing basal anxiety and PTSD phenotype (due to light, novelty, or temporary single housing to assess their activity in the light phase). However, none of these potential stressors are associated with physical damage to the animals.
The proposed transgenic mouse lines do not display a behavioral phenotype (1), and are therefore not associated with any expected discomfort.

References

1. [redacted]

Explain why these effects may emerge.

The cause of the stress of the mice is either primarily physical in nature (in case of foot shock, restraint stress, and blood sampling), or novelty/light-induced (in case of behavioral testing). These stressors are however necessary for these experiments to succeed.

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

Although some stressors are inherent to the experimental design, we will take precautionary measures to minimize all other potential causes of (additional) stress to the animals, e.g., by habituation to reverse cycle housing, and only partial cleaning of the housing cages to retain hierarchy (and thereby prevent fighting to re-establish this hierarchy). Moreover, i.p. injections and tail blood collection will only be performed by experienced researchers to minimize discomfort.

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.

The criteria to take the animal out of the experiments are based on human observation of factors known as clear symptoms of pain/stress/discomfort and defined for humane end point detection*. Weight loss of more than 15% in one day is considered as a humane endpoint. Also (a combination of) general symptoms such as raised fur, hunched back (arched back), and poor coat conditions are considered as humane endpoints after which the animals should be euthanized.
*Standard human endpoints rodents: piloerection, loss of body weight (> 15%), immobility, poor self-care, tremor, self-damage, abnormal body posture, convulsions, tumors, elephant teeth.

Indicate the likely incidence.

It is unexpected that any of the animals reach the human end point over the course of the experiment. So far, the primary researcher has exposed ~120 mice to the PTSD-induction protocol without witnessing any physical adverse effects. The human endpoint was only applied once (after consulting a veterinarian), when a mouse was sacrificed because it suffered from severe wounds from excessive fighting with a dominant cage mate, but this occurred prior to PTSD-induction.

K. Classification of severity of procedures

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

The total (cumulative) discomfort of the animals is expected to be severe (due to exposure to the PTSD-induction protocol).

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.

The brains of the animals are needed to analyse the neuronal activation patterns present at the different stages of PTSD-development (questions #1-3), and the neurobiological make up of these cells, i.e., the aim of objective A.

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

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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 2	Type of animal procedure B. Characterization of PTSD-associated neuronal circuits

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

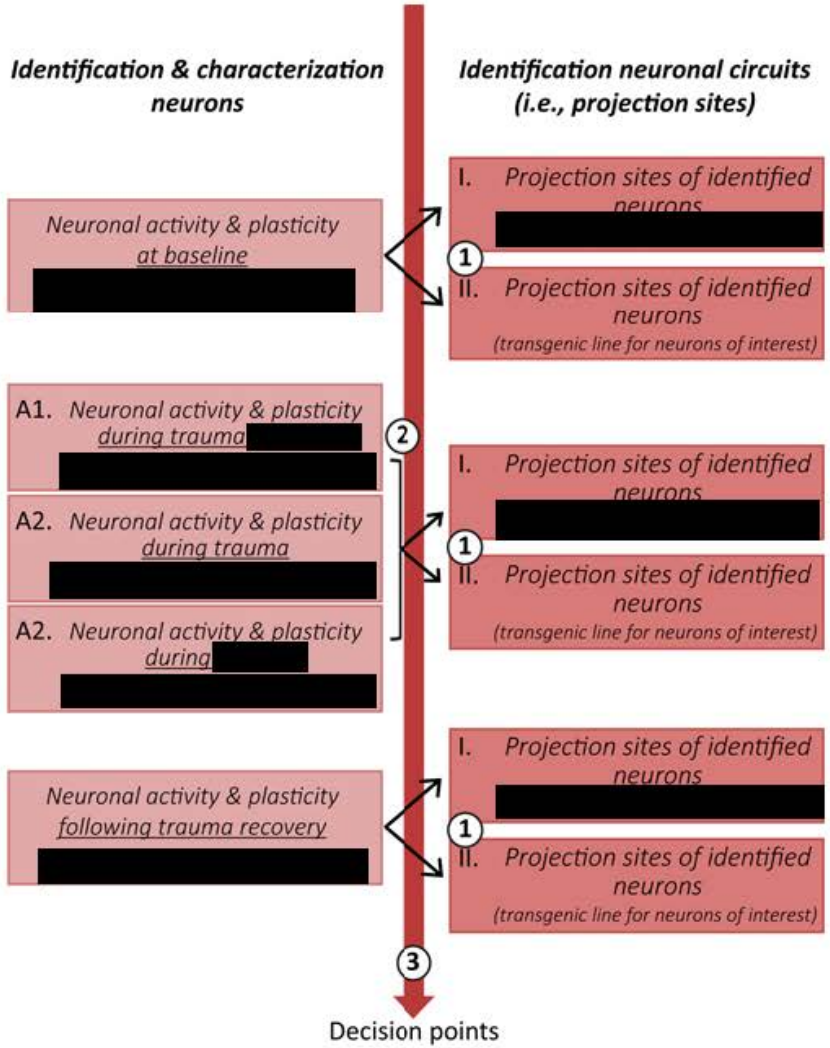
Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

In Part B of this proposal, we aim to identify the neuronal circuits involved in PTSD, by determining the projection sites of the neurons identified in Part A of this proposal. To do so, we will intracranially inject a fluorescent virus in the region of interest, and analyze its expression sites. Depending on the results of Part A, we will implement one of two approaches (Figure 3). Approach II will be followed if the neuronal subpopulation identified can be captured by a single, available transgenic mouse line, whereas we will use approach I if this is not the case. In case of the latter, the neuronal subpopulation identified in Part A of the proposal first has to be labeled again with Cre-recombinase expression, before projection sites can be labeled by viral injection.

Experimental question #1:
Which neuronal circuit function *at baseline* is predictive of PTSD-development?

Experimental question #2:
Which neuronal circuit *response to trauma exposure* predicts PTSD-development?

Experimental question #3:
Which neuronal circuit function is associated with PTSD-pathology and resiliency *following trauma recovery*?



Decision points:

1. If the neuronal subpopulation identified in Part A can be captured by a single available transgenic mouse line, approach II will be followed to reduce the amount of discomfort and the number of animals required
2. Part A1 and A2 allow for the most selective identification of the neuronal subpopulation responsible for later PTSD-development; we will only continue working with the protocol best targeting this population for Part B
3. Following the completion of Part A, we will carefully consider (through power analyses) if the number of animals required for Part B of the proposal can be further reduced by taking the intermediate PTSD-phenotypes into consideration (by performing correlational analyses)

Figure 5. Experimental design of Part B of the proposal.

APPROACH I:

General design

This approach is very similar to that described in Part A of the proposal. We will expose two transgenic mouse lines, [redacted] mice, to a PTSD-induction protocol, existing of a trauma (electric shock) [redacted] PTSD-like behavior in [redacted] of animals. A week after PTSD-induction, animals will be tested in a set of behavioral tests ([redacted]) assessing PTSD-symptomatology, and their neuroendocrine function will be tested (corticosterone response to restraint stress); [redacted]. As in Part A, active and plastic neuronal circuits will either be labeled at 1) baseline, 2) in response to PTSD-induction (i.e., trauma and/or [redacted] exposure), or 3) following recovery (once the pathology has been established), to obtain insight into the neuronal circuits associated with PTSD, and PTSD-[redacted] animals will be compared. However, now the labeling of active/plastic neuronal circuits will occur by intracranial injection of Cre-dependent eYFP virus in the brain region of interest in these mice. [redacted]. Three weeks after the end of the experimental protocol, animals will be sacrificed by perfusion fixation and their brains analyzed for expression of the fluorescent marker to identify the projection sites (circuits) of the neurons displaying aberrant activation.

Primary outcome parameters

-Behavioral phenotype; [redacted]
-Neuroendocrine function; [redacted]
-Pattern of active and plastic neuronal circuits at baseline (question #1); [redacted]
-Pattern of active and plastic neuronal circuits in response to trauma & trigger exposure (question #2); [redacted]
-Pattern of active and plastic neuronal circuits following recovery (question #3); [redacted]

Justification

The set of behavioral output measures is critical for the classification of animals [redacted] PTSD-[redacted]. Moreover, since neuroendocrine abnormalities are associated with PTSD, we include these measures as well to try to associate them with aberrant neuronal circuits in PTSD. The differences in activated and/or plastic neuronal circuits between [redacted] and PTSD-like animals at baseline will inform us about neuronal circuits which activity constitutes a risk factor for PTSD-development. The differences in activated and/or plastic neuronal circuits between [redacted] and PTSD-like animals in response to trauma[redacted] exposure will inform us about neuronal circuits which activation and/or plasticity reflects an immediate marker of PTSD-risk in reaction to trauma exposure. The differences in activated and/or plastic neuronal circuits between [redacted] and PTSD-like animals after recovery informs us about those circuits which activity and/or plasticity is associated with PTSD-pathology, and is therefore a useful target for treatment.

APPROACH II:

General design
As described before we will characterize the neurobiological makeup of the activated and plastic neuronal populations (differentiating the PTSD-susceptible from [redacted] brain) and perform **qPCR,** immunohistochemistry, and *in situ* hybridization experiments on the brain slices acquired in Part A. These experiments will allow us to identify the neuronal subpopulations displaying aberrant activation and/or plasticity associated with PTSD. If the neuronal subpopulation identified in Part A, primarily exists of a single cell type that can be captured in an available transgenic mouse line, we could also trace their neuronal projections (and thus the neuronal circuit affected) by merely intracranially injecting specific Cre-lines for the neuronal subpopulation identified. This approach will provide us information on the projection sites of the genetically specified subpopulation.
This second approach will reduce the number of animals needed for these experiments, and reduce the amount of discomfort the animals experience, since PTSD-induction and behavioral testing are not necessary. However, this approach can only be followed when we are able to identify a selective subpopulation of cells (which can be characterized by a single neurobiological/genetic marker) to behave differently in PTSD-[redacted] animals, which is only one of the many outcomes possible from Part A.

Primary outcome parameters
-Neuronal circuits associated with the neuronal subpopulation identified in Experiment A.

Justification
The neuronal circuits identified are informative on the signaling circuits of the abnormally functioning neurons in PTSD, and thus the information flow they represent. These circuits could next be targeted to prevent/treat PTSD.

References
1. Bremner JD (2002). Neuroimaging studies in post-traumatic stress disorder. *Curr Psychiatry Rep* 4: 254-263.
2. Shin LM, Rauch SL, Pitman RK (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci* 1071: 67-79.
3. Peterson A, Thome J, Frewen P, Lanius RA (2014). Resting-state neuroimaging studies: a new way of identifying differences and similarities among anxiety disorders? *Can J Psychiatry* 59(6): 294-300.
4. Kim MJ, *et al.* (2011). The structural and functional connectivity of the amygdala: from normal emotion to pathological anxiety. *Behav Brain Res* 223(2): 403-410.

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

APPROACH I:

I. Intracranial injection
At the start of the experiment, transgenic mice will be intracranially injected in the region of interest identified in Part A of the proposal with an AAV-virus encoding a Cre-dependent yellow fluorescent protein. Prior to injection, all mice will receive analgesics (buprenorphine, subcutaneously

injected) followed by inhalation of isoflurane anesthesia. Mice will be positioned on a stereotaxic instrument and a midline incision is made across the top of the skull, the periosteum is cleaned, and the brain is leveled. Cold virus will be injected using a Hamilton syringe connected to a motorized nanoinjector. To allow diffusion of the solution into the brain tissue, the needle is left for an additional 5 min after the injection, and then very slowly removed. The skin was stitched and animals are allowed to recover for a week from the surgery, and will be daily checked for symptoms of adversity. One day post-surgery animals receive a second dose of analgesics (buprenorphine, subcutaneously injected) to reduce potential discomfort (i.e., pain).

II. Basal anxiety

Open Field Test.

To assess basal anxiety, mice will be tested in the open field test. This test is based on the animals' natural conflict between exploration of and the aversion against open, bright areas. The open field apparatus consists of a white Plexiglas box (50 x 50 x 40 cm) lightened with 120 lux. Each mouse will be placed in the corner of the apparatus to initiate a 10 min test session. Time spent in the center (the inner 25 x 25 cm), distance traveled in the center, number of visits to the center, and total distance traveled will be quantified using a camera mounted above the apparatus and analyzed by Ethovision software ([redacted]).

Elevated Plus Maze.

As a second test for basal anxiety, the elevated plus maze will be used, which is also makes use of the rodents' aversion of open spaces. The elevated plus maze comprises a central part (5 x 5 cm), two opposing open arms (30.5 x 5 cm), and two opposing Plexiglas closed arms (30.5 x 5 x 15 cm), elevated at a height of 53.5 cm and the open arms are illuminated with 6-9 lux. Mice are placed in one of the closed arms facing the center to initiate a 5 min test session. Time spent in the open arms, distance traveled in the open arms, and number of visits to the open arms will be quantified using a camera mounted above the apparatus and analyzed by Ethovision software ([redacted]).

III. PTSD-induction

For these studies, male, adult mice will be exposed to a well-established mouse PTSD model ([redacted]) to induce a PTSD-like phenotype [redacted] animals. The model begins on day 1, [redacted] in duration over 85 min at variable intervals, representing the "trauma". [redacted]

IV. Behavioral tests for PTSD identification (phenotyping)

Mice are tested in five behavioral tests to determine whether they developed PTSD, each assessing different aspects of PTSD-symptomatology:

[redacted]

Percentage risk assessment.

One of the features of PTSD tested for is the impairment in risk assessment. In PTSD patients this dysfunction often manifests itself as paranoia (4) and risky behavior demonstrated by high incidences of violence, drug abuse, or suicide (5,6).The PTSD-like mice also show risk assessment patterns consistent with an immediate or imminent danger in the face of a predator and not to an uncertain potential for risk. Mice normally engage in oriented information-gathering scanning from place of concealment and increases in stretch attend posture (7,8) in the absence of a predator, but in the presence of a predator this activity is reduced in favor of quick flight. Increased risk assessment is also associated with reduced anxiety (9).
We will measure risk assessment using the dark/light transfer test (1). The test apparatus consists of a box divided by a partition into two environments: a dark covered compartment (15 x 20 x 25 cm) and a brightly illuminated (1000–1100 lux) light compartment (30 x 25 x 25 cm). The compartments are connected by a small passage in the bottom center of the partition. The mice are placed in the dark compartment to initiate a 5 minutes test session. Time spent in the light zone, number of visits to the light zone and the latency entering the light zone will be quantified using a camera mounted above the apparatus and analyzed by ([redacted]). An additional arena of 3 cm lengthwise by 6 cm width-wise will be programmed into the software tracking measurements surrounding the opening of the light area. Time spent in the risk assessment area and the number of visits to the risk assessment area are measured. Percentage risk assessment time will be calculated as the amount of time spent in the risk assessment arena as a percentage of total time spent in the light area outside of the risk assessment zone. [redacted]

Latency to peak startle amplitude and pre-pulse inhibition.

Exaggerated startle is one of the DSMIV criteria for PTSD (10), reflecting hyperarousal in patients. Moreover, impaired pre-pulse inhibition, a measure of sensorimotor gating but also a test that requires attentional processes, has been reported for PTSD (11). To assess the animals' (latency to) peak startle and the amount of pre-pulse inhibition we here use an acoustic startle protocol. The proposed protocol is similar to those reported before (1,12). Briefly, mice are placed in a small Plexiglas cage on top of a vibration-sensitive platform in a sound-attenuated, ventilated chamber. A high-precision sensor, integrated into the measuring platform, detects movement. Two high-frequency loudspeakers inside the chamber produce all the audio stimuli. The acoustic startle response (ASR) session begins with 5 min acclimation to white background noise (70 dB) maintained through the whole session. Thirty-two startle stimuli (120 dB, 40 ms in duration with a randomly varying ITI of 12–30 s) are presented interspersed with an additional 40 startle stimuli randomly preceded by 40 ms prepulses of either 74 dB, 78 dB, or 82 dB. Maximal ASR and latency to peak startle amplitude are measured both in response to individually presented startle stimuli and in response to startle stimuli preceded by pre-pulses. Percentage pre-pulse inhibition (PPI) will be calculated as the percent difference between the maximal ASR (max G) [redacted]

Marble burying.

Marble burying was assessed to measure hypervigilance and overall anxiety in the animals (13). Mice are placed in a compartment illuminated by 10 lux with dimensions (30 × 27 × 26 cm) containing 5 cm autoclaved bedding with 20 marbles centrally arranged 4 by 5. Mice are then filmed for 25 min. Videos are scored by counting the number of unburied marbles every 5 minutes until the end of the test (14). [redacted]

Homecage locomotion.
Homecage locomotion is assessed using the observation (Phenotyper) cages. Mice are housed individually for 72 h, in which the first 24 h are considered habituation to the individual housing conditions. Measurements of general locomotion consist of two light and two dark cycles in the last 48 h collected at 10 min intervals (1). [redacted] This test models the sleeping problems PTSD patients suffer from (10). Most patients will reach a clinical setting initially due to insomnia or disturbing nightmares, which to date have no specific cure.

Evaluation of [redacted]

V. Assessment of neuroendocrine function
Based on the **suggested role of the stress hormone corticosterone in stress recovery (15,16) and the** observation of neuroendocrine abnormalities in PTSD-patients, be it either in basal state or following a challenge (17,18), we will also monitor neuroendocrine function in PTSD-[redacted] mice over the course of PTSD-development, and later correlate these measures with neuronal activation. This will inform us on the potential causal relationship between neuroendocrine signaling and brain function and the supposed potential of corticosterone administration as treatment for PTSD (19,20). Therefore, corticosterone levels will be assessed by tail bleed (10 µL) 12 times over the course of the experiment.
- Two repetitions of basal corticosterone measurements in the morning (at the circadian peak) and evening (at the circadian trough); both at the start and at the end of the experiment (total = 8 measurements)
- Stress response corticosterone levels will be assessed in response to trauma and [redacted] (total = 2 measurements)
- Stress response corticosterone levels will be assessed in response to restraint stress; both at the start and at the end of the experiment, once pathology has been established (total = 8 measurements)

VI. Restraint stress

To measure the **corticosterone** stress response and subsequent recovery, animals will be exposed to 25 min restraint stress in plastic restrainers. Plasma will be extracted from blood samples (10 µL) that are collected by tail bleed at four time points: under basal conditions, at 25 min (i.e., immediately when removed from the restrainer), 75 min, and 120 min following stress initiation. This exact protocol has been used before to show abnormal corticosterone responding to stress in PTSD animals (1)**,** and correlational analyses with the brain findings will inform us on the neural basis of these changes.

[redacted]

VIII. Sacrifice

For histological read-out of neuronal projections, animals will be sacrificed by an overdose of anesthesia followed by transcardial perfusion with saline and fixative. The total duration of the experiment will be maximally 3 months.

APPROACH II:

Animals will only be exposed to step I (Intracranial injection) and step VIII (Sacrifice) of the procedures described for Approach I.

References

1. [redacted]
2. [redacted]
3. [redacted]
4. [redacted]
5. Panagioti M, Gooding P, Tarrier N (2009). Post-traumatic stress disorder and suicidal behavior: a narrative review. *Clin Psychol Rev* 29: 471–482.
6. Najt P, Fusar-Poli P, Brambilla P (2011). Co-occurring mental and substance abuse disorders: A review on the potential predictors and clinical outcomes. *Psychiatry Res* 186: 159–164.
7. Blanchard DC, Blanchard RJ, Tom P, Rodgers RJ (1990) Diazepam changes risk assessment in an anxiety/defense test battery. *Psychopharmacology* 101: 511–518.
8. Blanchard RJ, Blanchard DC (1989). Antipredator defensive behaviors in a visible burrow system. *J Comp Psychol* 103: 70–82.
9. Adamec RE, Shallow T (1993). Lasting effects on rodent anxiety of a single exposure to a cat. *Physiol Behav* 54: 101–109.
10. American Psychiatric Association (1994). *Diagnostic and statistical manual of mental disorders (DSM IV)* (Washington, DC, American Psychiatric).

11. Grillon C, Morgan CA, Southwick SM, Davis M, Charney DS (1996). Baseline startle amplitude and prepulse inhibition in Vietnam veterans with posttraumatic stress disorder. *Psychiatry Res* 64: 169–178.
12. Neufeld-Cohen A, Tsoory MM, Evans AK, Getselter D, Gil S, Lowry CA, Vale WW, Chen A (2010). A triple urocortin knockout mouse model reveals an essential role for urocortins in stress recovery. *Proc Natl Acad Sci U S A* 107: 19020–19025.
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15. Het S, Schoofs D, Rohleder N, Wolf OT (2012). Stress-induced cortisol level elevations are associated with reduced negative affect after stress: indications for a mood-buffering cortisol effect. *Psychosom Med* 74(1): 23-32.
16. Hermans EJ, Henckens MJ, Joëls M, Fernández G (2014). Dynamic adaptation of large-scale brain networks in response to acute stressors. *Trends Neurosci* 37(6): 304-14.
17. Yehuda R (2001). Biology of posttraumatic stress disorder. *J Clin Psychiatry* 62 Suppl 17: 41-46.
18. Daskalakis NP, Lehrner A, Yehuda R (2013). Endocrine aspects of post-traumatic stress disorder and implications for diagnosis and treatment. *Endocrinol Metab Clin North Am* 42(3): 503-513.
19. Schelling G, Briegel J, Roozendaal B, Stoll C, Rothenhäusler HB, Kapfhammer HP (2001). The effect of stress doses of hydrocortisone during septic shock on posttraumatic stress disorder in survivors. *Biol Psychiatry* 50(12): 978-985.
20. Zohar J, Yahalom H, Kozlovsky N, Cwikel-Hamzany S, Matar MA, Kaplan Z, Yehuda R, Cohen H (2011). High dose hydrocortisone immediately after trauma may alter the trajectory of PTSD: interplay between clinical and animal studies. *Eur Neuropsychopharmacol* 21(11): 796-809.

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

Below one can find a description of the number of animals requested for the execution of these experiments. However, as described before, we will only pursue these experiments when the experiments in part A have provided clear leads to these manipulations. Therefore, only a subset of the requested animals will be used.
Moreover, when Approach II will be suited/sufficient to answer our research question (i.e., the function of which neuronal circuits is associated with PTSD-susceptibility), we will implement this approach to use a minimum amount of animals.
Furthermore, we will only pursue the most promising neuronal subpopulation for research question #2 as identified in Part A of the proposal, which could either be targeted by labeling active/plastic neurons over the entire PTSD-induction procedure (A.2.A), or over the trauma or [redacted] period specifically (A.2.B).
Finally, as mentioned before in Part A, we will explore the option of correlational analyses on the data acquired of all animals, instead of taking into acount the extreme phenotypes only. This would potentially reduce the amount of animals needed.

APPROACH I:
For now, we estimate that we need maximally 12 mice per group. We will calculate the precise group sizes per experiment using a power analyses,

based on data collected so far by us and others (also exploring correlational analyses). As only 25% of all trauma-exposed animals is expected to either display a PTSD-like phenotype or [redacted] phenotype, we have to expose 48 mice per group to end up with these group sizes.

The following groups, as introduced in the project proposal, will be assessed:

[redacted]
1. PTSD-induction [redacted] at baseline (question #1)
2. PTSD-induction [redacted] at trauma+[redacted] (question #2a1)
   or PTSD-induction [redacted] at trauma (question #2a2)
   or PTSD-induction [redacted] (question #2a2)
3. PTSD-induction [redacted] at end (question #3)
   = 3x 48 mice, is 144 mice.

Furthermore, to be able to specifically identify the neuronal circuits related to the trauma/[redacted] exposure (instead of to e.g., [redacted] or novelty induced stress) also 12 control animals will be included for group 2, which are not exposed to PTSD-induction, but do receive the injection. Moreover, the inclusion of these control animals will inform us about the adaptive (adequate) neuronal circuit response to the trauma/[redacted] in the [redacted] animals. This all adds up to [redacted] in total.

[redacted]
4. PTSD-induction [redacted] at baseline (question #1)
5. PTSD-induction [redacted] at trauma[redacted] (question #2a1)
   or PTSD-induction [redacted] at trauma (question #2a2)
   or PTSD-induction [redacted] (question #2a2)
6. PTSD-induction[redacted] at end (question #3)

   = 3x 48 mice, is 144 mice.
Furthermore, to be able to specifically identify the neuronal circuits related to the trauma/[redacted] exposure (instead of to e.g., [redacted] or novelty induced stress) also 12 control animals will be included for group 5, which are not exposed to PTSD-induction, but do receive the injection. Moreover, the inclusion of these control animals will inform us about the adaptive (adequate) neuronal circuit response to the trauma[redacted] in the [redacted] animals. This all adds up to [redacted] mice in total.

APPROACH II:
Alternatively, we will implement approach II, for which we also estimate to need 12 mice per group. We will calculate the precise group sizes per experiment using a power analysis, based on data collected so far by us and others. However, for this approach no categorization as PTSD-like or [redacted] will be necessary, and 12 animals per group will be sufficient. Based on the findings of the experiments described in Part A of this proposal, we will use the corresponding transgenic mouse line to do these experiments. We have the following transgenic mouse lines available which are suitable for approach II:

*PV-Cre (parvalbumin (GABAergic) neurons)
*SS-Cre (somatostatin (GABAergic) neurons)
*Nex-Cre (glutamatergic neurons)
*Dlx5/6-Cre (GABAergic neurons)
*ePet-Cre (serotonergic neurons)
*Dat-Cre (dopaminergic neurons)
*Slc6a2-Cre (noradrenergic neurons)
*Ntsr1-Cre (neurotensin receptor neurons)
*Drd3-Cre (dopamine receptor neurons)
*CRFR1-Cre (CRF receptor 1 neurons)
*CRFR2-Cre (CRF receptor 2 neurons)
Since Part A of the proposal describes 6 separate experiments, with 6 potentially promising and distinct outcomes, we will maximally need 6 * 12 = 72 mice for this approach.

Thus, depending on the results of Part A of the proposal we will need maximally 312 mice when pursuing approach I, and maximally 72 mice when pursuing approach II.

B. The animals

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

APPROACH I:
The mouse is the lowest animal species in which we are able to model human psychiatric disorders. Because of the complexity of the brain and symptomatology to diagnose these disorders, it is impossible to use lower-order animals or organs. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and type of trauma exposure, and all pre-, peri-, and post-trauma factors potentially influencing an eventual disease outcome. This is not possible in humans.
Moreover, the proposed PTSD-protocol has been validated in mice. Here, we propose to use mice from two specific transgenic mouse lines; [redacted] Both transgenic lines label a distinct set of neurons and have their own characteristics in terms of selectivity (background labeling) and sensitivity ([redacted]), and therefore of added value to each other. Moreover, PTSD in human patients has been linked to both abnormal neural activity (3,4) and connectivity - reflecting plasticity - (5,6), making it necessary to target both processes independently.

APPROACH II:

The mouse is the lowest animal species which brain still resembles the human brain in terms of functional and structural connectivity. Therefore, they are also the lowest animal species in which the proposed neuronal tracing studies can be performed. Moreover, the transgenic mouse lines are chosen for their specific expression of the Cre-recombinase enzyme in the neuronal population of interest, allowing us to target these specific subclasses of neurons specifically. These models are also unprecedented.

References:

1. [redacted]
2. Root CM, Denny CA, Hen R, Axel R (2014). The participation of cortical amygdala in innate odour-driven behaviour. *Nature* 515: 269-275.
3. Bremner JD (2002). Neuroimaging studies in post-traumatic stress disorder. *Curr Psychiatry Rep* 4: 254-263.
4. Shin LM, Rauch SL, Pitman RK (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Ann N Y Acad Sci* 1071: 67-79.
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6. Kim MJ, *et al.* (2011). The structural and functional connectivity of the amygdala: from normal emotion to pathological anxiety. *Behav Brain Res* 223(2): 403-410.

Species	Origin	Maximum number of animals	Life stage
Mouse	own breeding	312	adult (> 8 weeks old)

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
Approach I: The mouse is the lowest animal species in which we are able to model human psychiatric disorders. Because of the complexity of the brain and symptomatology to diagnose these disorders, it is impossible to use lower-order animals or organs. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and type of trauma exposure, and all pre-, peri-, and post-trauma factors potentially influencing an eventual disease outcome. This is not possible in humans.*Approach II:* The mouse is the lowest animal species in which we are able to perform neuronal tracing studies, as its structural connectivity patterns resemble those of the human brain. This is not possible in other models.

Reduction
Approach I: The requested amount of animals (based on a group size of n = 12) is currently thought to be needed for statistical reliable conclusions and the minimal group size one can work with. As only a subset of the total amount of trauma-exposed animals will develop PTSD (25%) [redacted] the total group size has to be (4x) larger. However, as mentioned before, following the completion of Part A,
we will explore the option of correlational analyses on the data acquired of all animals, instead of taking into acount the extreme phenotypes only. This would potentially reduce the amount of animals needed. *Approach II:* However, to reduce the number of animals, we will use specific transgenic mouse lines for neuronal tracing when ever possible. These animals will only serve to define the structural projections (circuits) of a neuronal subpopulation of interest. As no behavioral assessment and categorization is required in this case, a group size of n = 12 will be sufficient.

Refinement
Approach I: The experiments will be carried out with the least discomfort possible. However, exposure to electrical shocks (and the discomfort caused by them) is critical to the induction of PTSD, whereas the intracranial injection is necessary for neuronal tracing and therefore unavoidable.

Approach II: However, to reduce any adverse effects as a consequence of the PTSD-induction procedure and repeated handling of the animals, we will use specific transgenic mouse lines for tracing whenever possible. These animals will only be exposed to the intracranial injection of the virus, without any further behavioral testing.

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

APPROACH I:
Analgesic will be administered both prior to surgery (intracranial injection) and one day post-surgery to prevent any pain, whereas the mice will be anesthesized during surgery using isoflurane. During recovery, animals will be monitored closely for signs of discomfort and checked daily to be able to detect Human End Point conditions.

Although the PTSD-induction procedure will be aversive to the animals, no physical adverse effects are expected. Animals will be monitored closely for signs of discomfort and checked daily to be able to detect Human End Point conditions. Handling of the animals will be minimized to reduce human intervention that may cause stress for the animals. Also, habituation to the housing at a reverse day/night rhythm will be applied. Perfusion will take place under deep anesthesia to minimize adverse effects.

APPROACH II:
Analgesic will be administered both prior to surgery (intracranial injection) and one day post-surgery to prevent any pain, whereas the mice will be anesthesized during surgery using isoflurane. During recovery, animals will be monitored closely for signs of discomfort and checked daily to be able to detect Human End Point conditions. Animals will be group housed to reduce any additional stress.

Perfusion will take place under deep anesthesia to minimize adverse effects.

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.

N/A

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?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

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

[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.

During surgery, all animals are anesthetized using isoflurane (inhalation).
All animals will receive a subcutaneous injection of buprenorphine (0.2 mg/kg) half an hour prior to the start of surgery, as well as the next morning (post-surgery). This analgesic is chosen for its long lasting analgesic effects, and repeated (2x) administration is therefore expected to be sufficient to reduce pain. Nevertheless, all animals will be daily checked for a week during recovery to monitor any symptoms of discomfort.

I. Other aspects compromising the welfare of the animals

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

I. Other aspects compromising the welfare of the animals

APPROACH I: The mice will experience severe discomfort during the PTSD-induction procedure, moderate stress during the restraint stress session, mild discomfort due to the [redacted] and tail blood collection, and very mild psychological discomfort in the behavioral tests assessing basal anxiety and PTSD phenotype (due to light, novelty, or temporary single housing to assess their activity in the light phase). However, none of these potential stressors are associated with physical damage to the animals. Moreover, they will experience moderate stress from the surgery due to the anesthesia.
APPROACH II: The mice will experience moderate stress from the surgery due to the anesthesia.

Explain why these effects may emerge.

The cause of the stress of the mice is either primarily physical in nature (in case of surgery, foot shock, restraint stress, and blood sampling), or novelty/light-induced (in case of behavioral testing). These stressors are however necessary for these experiments to succeed.

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

Although some stressors are inherent to the experimental design, we will take precautionary measures to minimize all other potential causes of (additional) stress to the animals, e.g., by using analgesics and anesthetics, habituation to reverse cycle housing, and only partial cleaning of the housing cages to retain hierarchy (and thereby prevent fighting to re-establish this hierarchy). Moreover, i.p. injections and all other procedures will only be performed by experienced researchers to minimize stress.

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.

No side effect of the intracranial injection procedure are expected.
The criteria to take the animal out of the experiments are based on human observation of factors known as clear symptoms of pain/stress/discomfort and defined for humane end point detection*. Weight loss of more than 15% in one day is considered as a humane endpoint. Also (a combination of) general symptoms such as raised fur, hunched back (arched back), and poor coat conditions are considered as humane endpoints after which the animals should be euthanized.

*Standard human endpoints rodents: piloerection, loss of body weight (> 15%), immobility, poor self-care, tremor, self-damage, abnormal body posture, convulsions, tumors, elephant teeth.

Indicate the likely incidence.

It is unexpected that any of the animals reach the human end point over the course of the experiment. So far, the primary researcher has exposed ~120 mice to the PTSD-induction protocol without witnessing any physical adverse effects. Also intracranial injections have been executed dozens of times by the primary researcher without any major side effects.
The human endpoint was only applied once in these experiments (after consulting a veterinary), when a mouse was sacrificed because it suffered from severe wounds from excessive fighting with a dominant cage mate, but this occurred prior to PTSD-induction and was thus unrelated to the proposed paradigm.

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).

APPROACH I:
The total (cumulative) discomfort of the animals is expected to be severe.

APPROACH II:
The total (cumulative) discomfort of the animals is expected to be moderate.

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.

The brains of the animals are needed to analyse the neuronal circuits labeled that are associated with PTSD-development (questions #1-3); this is in fact the primary outcome measure of Part B.

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

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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 3	Type of animal procedure C. Manipulation of PTSD-associated neuronal circuits

2 Description of animal procedures

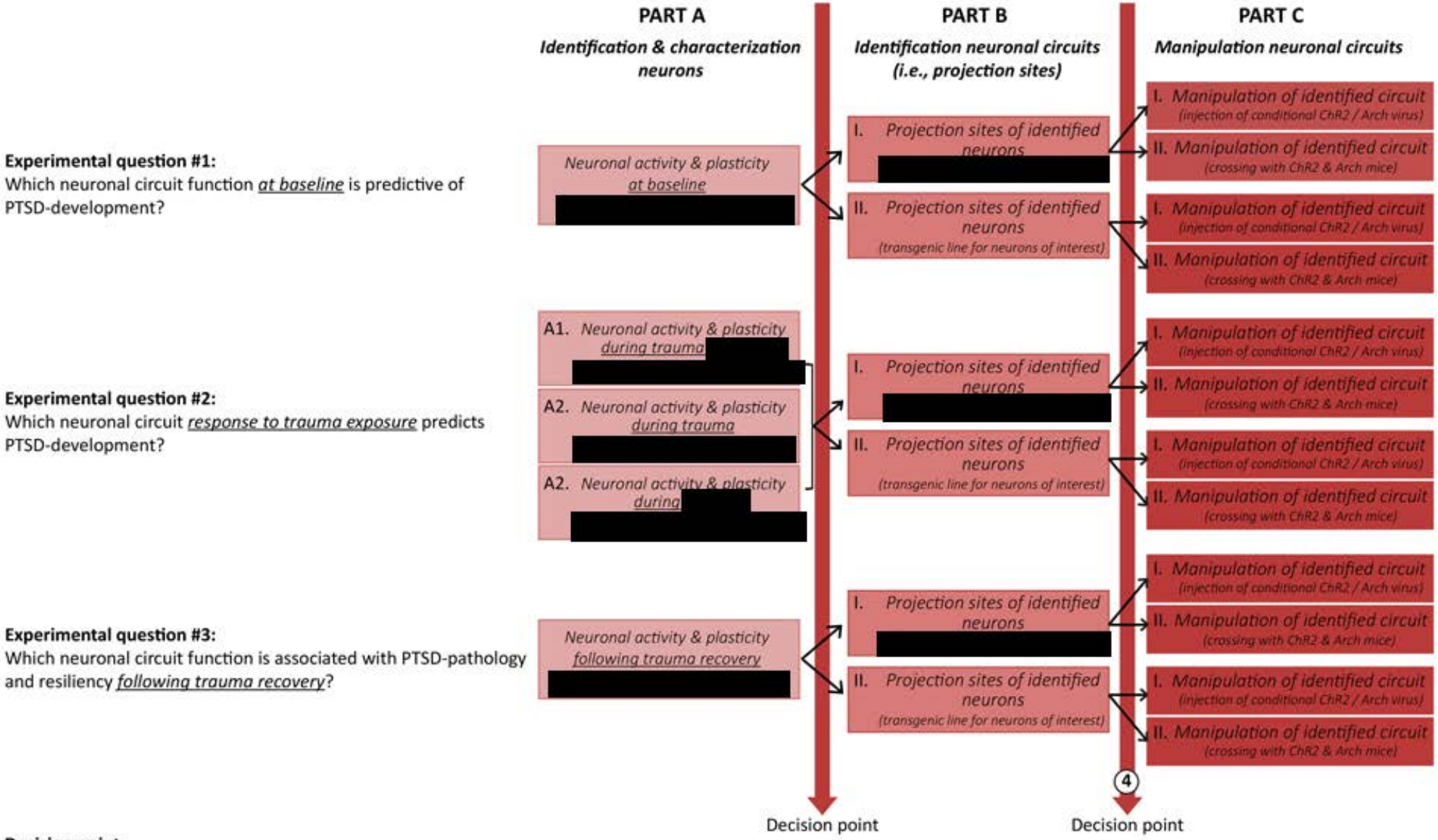
A. Experimental approach and primary outcome parameters

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

General design

To manipulate the identified neuronal circuits (in Part B) we will make use of optogenetics, a technique in which the activity of genetically modified neuronal populations can be manipulated by light. Depending on the results of Part B of this proposal, we will follow one of two approaches. Firstly (Approach I), we can sensitize our neuronal population of interest to light by the intracranial injection of a viral vector expressing the opsin, in transgenic mouse lines expressing Cre-recombinase in our neuronal population of interest. This approach has the major benefit of site-specific expression of the opsin, without any confounding expression elsewhere in the brain. However, it will require a substantial amount of neurons to be affected, so if the neuronal subpopulation of target (as identified in Part A of the proposal) is only sparsely present (or suboptimally targeted by viral injection), this approach will be not optimal and we will refer to another approach. The second approach (Approach II) is the sensitization of our neuronal population of interest to light by crossbreeding conditional channelrhodopsin (ChR2) and archaerhodopsin (Arch) mice with the transgenic Cre-lines for our neuronal population of interest. In this scenario, all Cre-expressing neurons throughout the brain will express the opsin - ensuring optimal sensitivity - but this goes at the cost of selectivity. Depending on the results from Part A & B of this proposal we will choose the most optimal approach for targeting our neuronal circuits of interest. In general, if the neuronal subpopulation and projection sites (i.e. circuit) are very widespread, approach I will be preferred to ensure specificity of the manipulation. When they are very locally focused, approach II is preferred.

Once we have established a neuronal subpopulation sensitized to light, we will need to implant optic fibers for local light delivery.
Targeting the neuronal circuits associated with PTSD, we will try to mimic the [redacted] brain either at baseline (question #1), immediately following trauma exposure (question #2), or following recovery (question #3), to see if we can treat the mice and reduce the PTSD-like phenotype.



Decision point:

④ In case the neurons whose circuit will be manipulated in Part C are highly abundant and focally concentrated, we will follow approach I and intracranially inject them for local opsin expression; if this is not the case, the offspring will be tested of the mouse line used for Part B of the proposal crossed with conditional ChR2 and Arch mice, to ensure sufficient levels of opsin expression

Figure 6. Experimental design of Part C.

Primary outcome measures
- Behavioral phenotype; [redacted]
- Neuroendocrine function; [redacted]
- Potential reduction in PTSD-incidence following photostimulation at baseline (question #1)
- Potential reduction in PTSD-incidence following photostimulation after trauma & trigger exposure (question #2)
- Potential reduction in PTSD-like symptoms following photostimulation after recovery (question #3)

Justification
The set of behavioral output measures is critical for the classification of animals [redacted] PTSD-[redacted]. Moreover, since neuroendocrine abnormalities are associated with PTSD, we include these measures as well to try to associate them with a potential reduction in PTSD-like phenotype. Optogenetic manipulation is necessary to provide causal evidence for the link between the abnormal function of the identified neuronal circuits and PTSD-incidence following trauma-exposure. These manipulations will provide us ultimately with tools to either prevent, immediately treat (and thereby prevent), and treat PTSD.

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

I. Surgery: Fiberoptic placement (Approach I & II) and intracranial injection of virus (Approach I)
At the start of the experiment, transgenic mice will undergo surgery, for which they will receive analgesics (buprenorphine, subcutaneously injected) followed by inhalation of isoflurane anesthesia. During surgery, mice will be intracranially injected in the region of interest with an AAV-virus encoding a Cre-dependent opsin (either ChR2 or Arch) in Approach I, but not in Approach II. Briefly, mice will be positioned on a stereotaxic instrument and a midline incision is made across the top of the skull, the periosteum is cleaned, and the brain is leveled. Cold virus will be injected using a Hamilton syringe connected to a motorized nanoinjector. To allow diffusion of the solution into the brain tissue, the needle is left for an additional 5 min after the injection, and then very slowly removed.
All animals will be implanted with 2 fiberoptic cannula's for local light delivery. In Approach I, this will happen immediately following the viral injection. In Approach II, he preparation of the animal will be exactly the same as described for Approach I. Two fiberoptic cannula's will be slowly inserted in the brain, and are secured using the C&B-Metabond kit and Jet acrylic dental cement.
Following surgery, mice are allowed to recover for a week, and will be daily checked for symptoms of discomfort. They will receive a second dose of analgesic (buprenorphine, subcutaneously injected) 1 day after the surgery.

II. Basal anxiety
Open Field Test.
To assess basal anxiety, mice will be tested in the open field test. This test is based on the animals' natural conflict between exploration of and the aversion against open, bright areas. The open field apparatus consists of a white Plexiglas box (50 x 50 x 40 cm) lightened with 120 lux. Each mouse

will be placed in the corner of the apparatus to initiate a 10 min test session. Time spent in the center (the inner 25 x 25 cm), distance traveled in the center, number of visits to the center, and total distance traveled will be quantified using a camera mounted above the apparatus and analyzed by Ethovision software ([redacted]).

Elevated Plus Maze.

As a second test for basal anxiety, the elevated plus maze will be used, which is also makes use of the rodents' aversion of open spaces. The elevated plus maze comprises a central part (5 x 5 cm), two opposing open arms (30.5 x 5 cm), and two opposing Plexiglas closed arms (30.5 x 5 x 15 cm), elevated at a height of 53.5 cm and the open arms are illuminated with 6-9 lux. Mice are placed in one of the closed arms facing the center to initiate a 5 min test session. Time spent in the open arms, distance traveled in the open arms, and number of visits to the open arms will be quantified using a camera mounted above the apparatus and analyzed by Ethovision software (Noldus, Wageningen, Netherlands).

III. PTSD-induction

For these studies, male, adult mice will be exposed to a well-established mouse PTSD model ([redacted]) to induce a PTSD-like phenotype in [redacted] animals. The model begins on day 1, [redacted], in which mice receive 14 shocks of 1 mA, 1 s in duration over 85 min at variable intervals, representing the "trauma". [redacted]

IV. Behavioral tests for PTSD identification (phenotyping)

Mice are tested in five behavioral tests to determine whether they developed PTSD, each assessing different aspects of PTSD-symptomatology: [redacted] Moreover, to answer research question #3, i.e., whether we can treat PTSD and reduce its symptoms, part of the animals will be retested in these behavioral tasks following the manipulation.

Percentage risk assessment.

One of the features of PTSD tested for is the impairment in risk assessment. In PTSD patients this dysfunction often manifests itself as paranoia (4) and risky behavior demonstrated by high incidences of violence, drug abuse, or suicide (5,6).The PTSD-like mice also show risk assessment patterns consistent with an immediate or imminent danger in the face of a predator and not to an uncertain potential for risk. Mice normally engage in oriented information-gathering scanning from place of concealment and increases in stretch attend posture (7,8) in the absence of a predator, but in the presence of a predator this activity is reduced in favor of quick flight. Increased risk assessment is also associated with reduced anxiety (9).

We will measure risk assessment using the dark/light transfer test (1). The test apparatus consists of a box divided by a partition into two environments: a dark covered compartment (15 x 20 x 25 cm) and a brightly illuminated (1000–1100 lux) light compartment (30 x 25 x 25 cm). The compartments are connected by a small passage in the bottom center of the partition. The mice are placed in the dark compartment to initiate a 5 minutes test session. Time spent in the light zone, number of visits to the light zone and the latency entering the light zone will be quantified using a camera mounted above the apparatus and analyzed by ( ). An additional arena of 3 cm lengthwise by 6 cm width-wise will be programmed into the software tracking measurements surrounding the opening of the light area. Time spent in the risk assessment area and the number of visits to the risk assessment area are measured. Percentage risk assessment time will be calculated as the amount of time spent in the risk assessment arena as a percentage of total time spent in the light area outside of the risk assessment zone.

Latency to peak startle amplitude and pre-pulse inhibition.
Exaggerated startle is one of the DSMIV criteria for PTSD (10), reflecting hyperarousal in patients. Moreover, impaired pre-pulse inhibition, a measure of sensorimotor gating but also a test that requires attentional processes, has been reported for PTSD (11). To assess the animals' (latency to) peak startle and the amount of pre-pulse inhibition we here use an acoustic startle protocol. The proposed protocol is similar to those reported before (1,12). Briefly, mice are placed in a small Plexiglas cage on top of a vibration-sensitive platform in a sound-attenuated, ventilated chamber. A high-precision sensor, integrated into the measuring platform, detects movement. Two high-frequency loudspeakers inside the chamber produce all the audio stimuli. The acoustic startle response (ASR) session begins with 5 min acclimation to white background noise (70 dB) maintained through the whole session. Thirty-two startle stimuli (120 dB, 40 ms in duration with a randomly varying ITI of 12–30 s) are presented interspersed with an additional 40 startle stimuli randomly preceded by 40 ms prepulses of either 74 dB, 78 dB, or 82 dB. Maximal ASR and latency to peak startle amplitude are measured both in response to individually presented startle stimuli and in response to startle stimuli preceded by pre-pulses. Percentage pre-pulse inhibition (PPI) will be calculated as the percent difference between the maximal ASR (max G) to startle stimuli preceded by pre-pulses compared to that without.

<u>Marble burying.</u>
Marble burying was assessed to measure hypervigilance and overall anxiety in the animals (13). Mice are placed in a compartment illuminated by 10 lux with dimensions (30 × 27 × 26 cm) containing 5 cm autoclaved bedding with 20 marbles centrally arranged 4 by 5. Mice are then filmed for 25 min. Videos are scored by counting the number of unburied marbles every 5 minutes until the end of the test (14).

Homecage locomotion.
Homecage locomotion is assessed using the observation (Phenotyper) cages. Mice are housed individually for 72 h, in which the first 24 h are considered habituation to the individual housing conditions. Measurements of general locomotion consist of two light and two dark cycles in the last 48 h collected at 10 min intervals (1). . This test models the sleeping problems PTSD patients suffer from (10). Most patients will reach a clinical setting initially due to insomnia or disturbing nightmares, which to date have no specific cure.

V. Assessment of neuroendocrine function

Based on the **suggested role for corticosterone in stress recovery (15,16) and the** observation of neuroendocrine abnormalities in PTSD-patients, be it either in basal state or following a challenge (17,18), we will also monitor neuroendocrine function in PTSD-[redacted] mice over the course of PTSD-development, and later correlate these measures with neuronal activation. This will inform us on the potential causal relationship between neuroendocrine signaling and brain function and the supposed potential of corticosterone administration as treatment for PTSD (19,20). Therefore, corticosterone levels will be assessed by tail bleed (10 µL) 12 times over the course of the experiment.

- Two repetitions of basal corticosterone measurements in the morning (at the circadian peak) and evening (at the circadian trough); both at the start and at the end of the experiment (total = 8 measurements)
- Stress response corticosterone levels will be assessed in response to trauma and [redacted] (total = 2 measurements)
- Stress response corticosterone levels will be assessed in response to restraint stress; both at the start and at the end of the experiment, once pathology has been established (total = 8 measurements)

VI. Restraint stress

To measure the corticosterone stress response and subsequent recovery, animals will be exposed to 25 min restraint stress in plastic restrainers. Plasma will be extracted from blood samples (10 µL) that are collected by tail bleed at four time points: under basal conditions, at 25 min (i.e., immediately when removed from the restrainer), 75 min, and 120 min following stress initiation. This exact protocol has been used before to show abnormal corticosterone responding to stress in PTSD-like animals (1), and correlational analyses with the brain findings will inform us on the neural basis of these changes.

VII. [redacted]

VIII. Optogenetic photostimulation

To manipulate the activity and signaling of neuronal circuits of interest, we will apply photostimulation for each experimental group at different time points throughout the experimental paradigm (see statistics section for the exact groups and manipulation). Mice will either receive photostimulation at baseline (question #1), immediately following PTSD-induction (question #2), or following recovery (question #3). For questions #1 & #2 the incidence of PTSD-development of mice receiving photostimulation will be the main output measure and will be compared with a control group to determine the effects of photostimulation. For question #3, mice will first be phenotyped [redacted] PTSD-[redacted] and subsequently subjected to photostimulation. Then, mice will be retested in the behavioral paradigm, to see whether PTSD-symptomatology in the PTSD-[redacted] animals was reduced or PTSD-incidence decreased as a consequence of photostimulation. A control group will be included to control for the effects of time and repeated testing. In all cases, the experimental group exists of animals expressing the opsin and are thus sensitive to photostimulation, whereas the control group exists of their littermates, which are undergoing the exact same procedures, but are not expressing the opsin - since they have a different genotype - and are thus insensitive to photostimulation.

VIV. Sacrifice

For histological read-out of neuronal projections, animals will be sacrificed by an overdose of anesthesia followed by transcardial perfusion with saline and fixative. The total duration of the experiment will be maximally 3 months.

References

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2. [redacted]
3. [redacted] 3.
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Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Below one can find a description of the number of animals requested for the execution of these experiments. However, as described before, we will only pursue these experiments in the most promising groups, based on the results of the experiments described in Part A & B of this proposal. Therefore, only a subset of the requested animals will be used.
As mentioned before in Part A, we will explore the option of correlational analyses on the data acquired of all animals, instead of taking into acount the extreme phenotypes only. This would potentially reduce the amount of animals needed.

For now, we estimate that we need maximally 12 mice per group. We will calculate the precise group sizes per experiment using a power analyses, based on data collected so far by us and others (also exploring correlational analyses). As only 25% of all trauma-exposed animals is expected to either display a PTSD[redacted] phenotype or [redacted] phenotype, we have to expose 48 mice per group to end up with these group sizes.

To determine the effects of neuronal circuit (de)activation, we will compare the incidence of PTSD and PTSD-symptomatology in an experimental group with that of a control group. Therefore, double the amount of animals (n = 96) is necessary to determine the effect of our manipulation.

The following groups could be assessed to answer experimental question #1, and will receive (repeated) photostimulation at baseline:
1. [redacted]
2. [redacted]
or:
3. Specific transgenic line (as specified in Part B of the proposal) targeting aberrant neuronal circuit <u>activation</u> (as identified initially in the [redacted] mice) associated with PTSD
(PV-Cre, SS-Cre, Nex-Cre, Dlx5/6-Cre, ePet-Cre, Dat-Cre, Slc6a2-Cre, Ntsr1-Cre, Drd3-Cre, CRFR1-Cre, CRFR2-Cre)

4. Specific transgenic line (as specified in Part B of the proposal) targeting aberrant neuronal circuit plasticity (as identified initially in the [redacted] mice) associated with PTSD
(PV-Cre, SS-Cre, Nex-Cre, Dlx5/6-Cre, ePet-Cre, Dat-Cre, Slc6a2-Cre, Ntsr1-Cre, Drd3-Cre, CRFR1-Cre, CRFR2-Cre)
adding up to 2*96 = 192 mice to answer experimental question #1.

The following groups could be assessed to answer experimental question #2, and will receive photostimulation immediately following PTSD-induction:
5. Specific transgenic line (as specified in Part B of the proposal) targeting aberrant neuronal circuit activation associated with PTSD
(PV-Cre, SS-Cre, Nex-Cre, Dlx5/6-Cre, ePet-Cre, Dat-Cre, Slc6a2-Cre, Ntsr1-Cre, Drd3-Cre, CRFR1-Cre, CRFR2-Cre)
6. Specific transgenic line (as specified in Part B of the proposal) targeting aberrant neuronal circuit plasticity associated with PTSD
(PV-Cre, SS-Cre, Nex-Cre, Dlx5/6-Cre, ePet-Cre, Dat-Cre, Slc6a2-Cre, Ntsr1-Cre, Drd3-Cre, CRFR1-Cre, CRFR2-Cre)

Alternatively, if it is the case that certain neuronal subpopulations displaying aberrant activity and/or plasticity during the trauma or trigger session are best predictive of later PTSD-development, but cannot be captured by one of the listed transgenic lines (groups 5 & 6) we will also target these neuronal circuits in a later stage of PTSD development to reduce PTSD-symptomatology:
7. [redacted] prior to PTSD induction
[redacted] prior to trauma exposure
[redacted]
8. [redacted] prior to PTSD induction
[redacted] prior to trauma exposure
[redacted]
adding up to maximally 2*96 = 192 mice to answer experimental question #2.

The following groups could be assessed to answer experimental question #3, and will receive (repeated) photostimulation following recovery:
13. [redacted] after recovery
14. [redacted] after recovery
or:
15. Specific transgenic line (as specified in Part B of the proposal) targeting aberrant neuronal circuit activation associated with PTSD
(PV-Cre, SS-Cre, Nex-Cre, Dlx5/6-Cre, ePet-Cre, Dat-Cre, Slc6a2-Cre, Ntsr1-Cre, Drd3-Cre, CRFR1-Cre, CRFR2-Cre)
16. Specific transgenic line (as specified in Part B of the proposal) targeting aberrant neuronal circuit plasticity associated with PTSD
(PV-Cre, SS-Cre, Nex-Cre, Dlx5/6-Cre, ePet-Cre, Dat-Cre, Slc6a2-Cre, Ntsr1-Cre, Drd3-Cre, CRFR1-Cre, CRFR2-Cre)
adding up to 2*96 = 192 mice to answer experimental question #3.

In total, this adds up to maximally (192+192+192 =) 576 mice

B. The animals

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

The mouse is the lowest animal species in which we are able to model human psychiatric disorders. Because of the complexity of the brain and symptomatology to diagnose these disorders, it is impossible to use lower-order animals or organs. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and type of trauma exposure, and all pre-, peri-, and post-trauma factors potentially influencing an eventual disease outcome. This is not possible in humans.
Moreover, the proposed PTSD-protocol has been validated in mice. Lastly, optogenetic manipulation of specific neuronal circuits (characterized by a certain neuronal marker or activity) is by far best developed in this model system, offering a wide variety of transgenic animals one can work with.

Species	Origin	Maximum number of animals	Life stage
Mouse	own breeding	576	adult (>8 weeks old)

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 mouse is the lowest animal species in which we are able to model human psychiatric disorders. Because of the complexity of the brain and symptomatology to diagnose these disorders, it is impossible to use lower-order animals or organs. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and type of trauma exposure, and all pre-, peri-, and post-trauma factors potentially influencing an eventual disease outcome. This is not possible in humans.
Moreover, the proposed PTSD-protocol has been validated in mice. Lastly, optogenetic manipulation of specific neuronal circuits (characterized by a certain neuronal marker or activity) is by far best developed in this model system, offering a wide variety of transgenic animals one can work with.
Reduction
The requested amount of animals (based on a group size of n = 12) is needed for statistical reliable conclusions and is the minimal group size one can work with. As only a subset of the total amount of trauma-exposed animals will develop PTSD (25%) and [redacted] the total group size has to be (4x) larger. However, as mentioned before, following the completion of Part A, we will explore the option of correlational analyses on the data acquired of all animals, instead of taking into acount the extreme phenotypes only. This would potentially reduce the amount of animals needed.

Refinement
The experiments will be carried out with the least discomfort possible. However, the exposure to electrical shocks (and the discomfort caused by them) is critical to the induction of PTSD, whereas surgery is required for our manipulation and therefore unavoidable.

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

Analgesic will be administered both prior to surgery (intracranial injection and implantation of the optic fibers) and one day after, to prevent any pain, whereas the mice will be anesthesized during surgery using isoflurane. During recovery, animals will be monitored closely for signs of discomfort and checked daily to be able to detect Human End Point conditions.
Although the PTSD-induction procedure will be aversive to the animals, no physical adverse effects are expected. Animals will be monitored closely for signs of discomfort and checked daily to be able to detect Human End Point conditions. Handling of the animals will be minimized to reduce human intervention that may cause stress for the animals. Also, habituation to the housing at a reverse day/night rhythm will be applied. Perfusion will take place under deep anesthesia to minimize adverse effects.

Repetition and Duplication

E. Repetition

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.

N/A

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?

[X] No
[] Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

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

[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

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.

During surgery, all animals are anesthetized using isoflurane (inhalation).
All animals will receive a subcutaneous injection of buprenorphine (0.2 mg/kg) half an hour prior to the start of surgery, as well as the next morning (post-surgery). This analgesic is chosen for its long lasting analgesic effects, and repeated (2x) administration is therefore expected to be sufficient to reduce pain. Nevertheless, all animals will be daily checked during recovery to monitor any symptoms of discomfort.

I. Other aspects compromising the welfare of the animals

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

The mice will experience moderate discomfort from the surgery due to the anesthesia.
The mice will experience severe discomfort during the PTSD-induction procedure, moderate stress during the restraint stress session, mild discomfort due to the evt. [redacted] and tail blood collection, and very mild psychological discomfort in the behavioral tests assessing basal anxiety and PTSD phenotype (due to light, novelty, or temporary single housing to assess their activity in the light phase). However, none of these potential stressors are associated with physical damage to the animals.

Explain why these effects may emerge.

The cause of the stress of the mice is either primarily physical in nature (in case of surgery, foot shock, restraint stress, and blood sampling), or novelty/light-induced (in case of behavioral testing). These stressors are however absolutely necessary for these experiments to succeed.

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

Although some stressors are inherent to the experimental design, we will take precautionary measures to minimize all other potential causes of (additional) stress to the animals, e.g., by using analgesics and anesthetics, habituation to reverse cycle housing, and only partial cleaning of the housing cages to retain hiearchy (and thereby prevent fighting to re-establish this hiearchy). Moreover, i.p. injections and all other procedures will only be performed by experienced researchers to minimize stress.

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.

No side effects of the intracranial injection and fiberoptic cannula placement are expected.
The criteria to take the animal out of the experiments are based on human observation of factors known as clear symptoms of pain/stress/discomfort and defined for humane end point detection*. Weight loss of more than 15% in one day is considered as a humane endpoint. Also (a combination of) general symptoms such as raised fur, hunched back (arched back), and poor coat conditions are considered as humane endpoints after which the animals should be euthanized.
*Standard human endpoints rodents: piloerection, loss of body weight (> 15%), immobility, poor self-care, tremor, self-damage, abnormal body posture, convulsions, tumors, elephant teeth.

Indicate the likely incidence.

It is unexpected that any of the animals reach the human end point over the course of the experiment. So far, the primary researcher has exposed ~120 mice to the PTSD-induction protocol without witnessing any physical adverse effects. Also intracranial injections and fiberoptic surgeries have been executed dozens of times by the primary researcher without any major side effects.
The human endpoint was only applied once in these experiments (after consulting a veterinary), when a mouse was sacrificed because it suffered from severe wounds from excessive fighting with a dominant cage mate, but this occurred prior to PTSD-induction and was thus unrelated to the proposed paradigm.

K. Classification of severity of procedures

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

The total (cumulative) discomfort of the animals is expected to be severe.

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.

The brains of the animals are needed for histological read-out of neuronal activation and opsin expression.

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

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer 2015-0090
2. Titel van het project: Neural correlates of post-traumatic stress disorder: natural resilience as key for intervention
3. Titel van de NTS: Onderzoek naar de hersenkenmerken die beschermen tegen post-traumatische stress stoornis
4. Type aanvraag:
   - ☐ nieuwe aanvraag projectvergunning
5. Contactgegevens DEC:
   - Naam DEC: RUDEC
   - Telefoonnummer contactpersoon: [redacted], bereikbaar op maandag, dinsdag, en donderdag van 9:00 tot 15:00 uur
   - Mailadres contactpersoon: [redacted]
6. Adviestraject:
   - ☐ ontvangen door DEC: 22-09-2015
   - ☐ aanvraag compleet
   - ☐ in vergadering besproken: 06-10-2015 en 03-11-2015
   - ☐ anderszins behandeld
   - ☐ termijnonderbreking(en) van 12-10-2015 tot 20-10-2015 en van 09-11-2015 tot 11-11-2015
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   - ☐ aanpassing aanvraag: 20-10-2015 en 11-11-2015
   - ☐ advies aan CCD: 24-11-2015
7. Eventueel horen van aanvrager
   - Datum
   - Plaats
   - Aantal aanwezige DEC-leden
   - Aanwezige (namens) aanvrager
   - Strekking van de vraag / vragen
   - Strekking van het (de) antwoord(en)
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag
8. Correspondentie met de aanvrager
   - Datum: 12-10-2015
   - Strekking van de vragen:

     Niet-technische samenvatting:

     -Deze NTS voldoet niet aan de gestelde criteria. De inhoud zou moeten aansluiten bij de fundamentele insteek van het project en minder speculeren over de mogelijke translationele waarde. Het taalgebruik is te ingewikkeld voor de doelgroep.

     -3.4 De vermelding van alleen ‘milde’ schokken is een onjuiste voorstelling van zaken voor een model dat de DEC als ernstig ongerief beschouwt.

-3.5 De vermelding van het cumulatieve ongerief voor de dieren ontbreekt. De beschrijving van het ongerief per handeling wordt hier gegeven maar is niet gevraagd.

Project Proposal:

-Er staat een storende spelfout in de hele aanvraag. De onderzoekers worden verzocht inedible te vervangen door indelible.

-3.1 Er bestaan vele diermodellen voor PTSS. De onderzoekers bestempelen het model dat zij willen gebruiken als 'well-established', maar twee van de drie referenties die zij geven betreffen kennelijk niet-geaccepteerde artikelen. Bovendien komt al het onderzoek uit hetzelfde laboratorium. De commissie verzoekt de onderzoekers beter te onderbouwen waarom zij dit een 'well-established' model achten, of anders deze classificatie te nuanceren.

-3.1 De gegeven achtergrond zou een verduidelijking moeten zijn van onderdeel 3.2: de doelstelling en de onderzoeksvragen van het project. De drie onderzoeksvragen zijn nog onvoldoende ingeleid bij onderdeel 3.1. Dit geldt met name voor de vragen 2 en 3. Wat is de noodzaak tot het doen van deze experimenten? Betreft het drie onafhankelijke onderzoeksvragen, of is er een verband c.q. onderlinge afhankelijkheid tussen de vragen? Wat bedoelen de onderzoekers precies met 'response to trauma exposure' en wat is het verschil met 'following trauma recovery'? Mag de DEC aannemen dat de onderzoekers hiermee niet bedoelen dat de dieren spontaan herstellen van PTSS? Zo ja, wat bedoelen zij dan wel?

-3.1 Er is een groot verschil tussen mannen en vrouwen in gevoeligheid voor PTSS: het risico op PTSS is tweemaal zo hoog bij vrouwen. Dit leidt tot uitdagingen waar het de validiteit van modellen betreft (Shansky, Neurobiol Stress 2015). Waarom willen de onderzoekers mannelijke dieren gebruiken? Indien zij menen dat dit geen consequenties heeft voor de extrapolatie van de resultaten naar de humane situatie worden zij verzocht dit duidelijk te onderbouwen.

-3.1 De fysiologie die ten grondslag ligt aan deze aanvraag is onderbelicht. Zien de onderzoekers een verband met de verschillende copingstyles in respons op stress? Welke betrokken circuits verwachten de onderzoekers mogelijk te vinden in dit project?

-3.1 De verwijzing naar de financiering van dit project is een aspect dat iets zegt over de haalbaarheid en het belang ervan in de ogen van vakgenoten, en hoort daarom bij onderdeel 3.2 en 3.3. De onderzoekers worden verzocht dit aan te passen.

-3.2 De onderzoekers worden verzocht hier alleen de doelstelling van het project inclusief de onderzoeksvragen te benoemen en dan in te gaan op de haalbaarheid van de doelstellingen. De beschrijving van de strategie (onderdelen A, B en C) hoort bij onderdeel 3.4. De laatste alinea is een beschrijving van het belang, en hoort daarom bij onderdeel 3.3.

-3.3 De onderzoekers willen met dit onderzoek een fundamentele vraag beantwoorden. Het wetenschappelijke belang hiervan is zeker zo groot als het maatschappelijke belang, maar blijft nu onderbelicht. Het onderzoek leidt mogelijk tot het identificeren van biomarkers in de hersenen die gevoeligheid voor PTSS voorspellen. Hoe kunnen deze biomarkers in de populatie bepaald worden waardoor zij van translationele waarde kunnen zijn? De onderzoekers worden verzocht dit beter te onderbouwen.

-3.4.1 Hebben de resultaten van onderzoeksvraag 1 geen invloed op het design van de experimenten om onderzoeksvraag 2 en 3 te beantwoorden? De onderzoekers worden gevraagd dit beter uit te leggen (zie ook de tweede vraag over 3.1). Tevens ziet de commissie

dit als een belangrijk go/no go moment voor de beide andere onderzoeksvragen. Zij verzoekt de onderzoekers dit op te nemen in de aanvraag of zich in eerste instantie te beperken tot onderzoeksvraag 1 en pas een vervolgaanvraag te schrijven indien er meer duidelijkheid is over de haalbaarheid en relevantie van de gekozen aanpak. De commissie heeft een voorkeur voor de tweede optie, maar houdt de mogelijkheid open dat de onderzoekers hun aanpak voldoende zullen kunnen onderbouwen om de huidige aanvraag te handhaven.
-3.4.2 Kunnen de onderzoekers met behulp van een tijdslijn per onderzoeksvraag aangeven op welk tijdstip welke handelingen zullen plaatsvinden?
-3.4.2 Uit de beschrijving van de milestones bij 3.4.3 lijkt het alsof de drie onderzoeksvragen na elkaar onderzocht zullen worden, terwijl dit niet blijkt uit figuur 1.
-3.4.2 Kunnen de onderzoekers duidelijker uitleggen hoe zij bij onderdeel A de neurobiologie gaan onderzoeken in het hersenweefsel? Welke technieken willen de onderzoekers daarvoor gebruiken, en denken zij dan ook aan in-situ hybridisatie of Q-PCR? Kunnen zij daarmee activatie of plasticiteit aantonen?
-3.4.2 Zou het misschien toch relevant kunnen zijn om onderdeel B uit te voeren indien er in onderdeel A geen specifiek gebied gevonden wordt?
-3.4.2 Kunnen de dieren na implantatie van de optische vezels zich nog voldoende vrij bewegen zonder dat dit invloed heeft op de uitkomst van de voorgesteld gedragstesten?
-3.4.3 Kunnen de onderzoekers de criteria specifieker benoemen voor beslispunt A en beslispunt B?

Description of Animal Procedures:
-De commissie is niet akkoord met de huidige ongeriefclassificatie van de dierexperimenten. De onvermijdbare en onvoorspelbare elektrische schokken die ook nog eens gecombineerd worden met huisvesting in fel licht en lawaai geven ernstig ongerief (zie bijlage VIII van Richtlijn 2010/63/EU). Alle dieren die PTSS inductie ondergaan ondervinden derhalve ernstig ongerief, onafhankelijk van het feit of zij PTSS ontwikkelen. Dat dieren die geen PTSS ontwikkelen DUS ook de schokken als minder ernstig hebben ervaren, mag niet zonder meer geconcludeerd worden.
-DAP1 vraag A1. Waarom willen de onderzoekers zowel het trauma als de trigger bestuderen? De rationale hiervoor is onvoldoende toegelicht.
-DAP1 vraag A2, IV: de beschreven bepaling van de neuroendocriene functie is onvoldoende om een goed beeld van de neuroendocriene functie van een dier te krijgen. Bovendien is de achtergrond van deze bepaling onvoldoende beschreven in het project. De handelingen leveren wel extra ongerief voor de dieren. De onderzoekers worden verzocht de noodzaak van deze bepaling te heroverwegen, of er een zodanige bepaling van te maken dat de uitkomsten te interpreteren zijn in het kader van dit experiment.
-DAP1 vraag A2. In het project proposal onderdeel 3.4.2 staat vermeld dat er immunohistochemische experimenten worden uitgevoerd om de neurobiologie van de gelabelde cellen te onderzoeken. Deze experimenten ontbreken in deze beschrijving. Kunt u toelichten hoe u de activatie of plasticiteit d.m.v. immunohistochemie (of wellicht in situ-hybridisatie of Q-PCR) kunt aantonen?
-DAP1 vraag A3. De argumentatie voor het toevoegen van 12 controledieren per experiment ontbreekt.

-DAP1 vraag D2. Tweemaal per week checken is te weinig voor een goede bepaling van humane eindpunten. Groepshuisvesting is een standaard procedure die niet speciaal wordt toegepast om het ongerief voor de dieren te verkleinen.
-DAP1 vraag J. Het humane eindpunt is gewichtsverlies van meer dan 15% in één dag (niet 15-20%). De commissie verzoekt de onderzoekers de zin 'All criteria that have been considered in the past … t/m … veterinarians at [redacted].weg te halen.
-DAP1 vraag K. De onderzoekers worden verzocht het cumulatief ongerief per dier in plaats van per handeling aan te geven.

De onderzoekers worden verzocht na te gaan welke opmerkingen over DAP1 ook van toepassing zijn op DAP2 en DAP3 en deze waar nodig aan te passen.

- Datum antwoord: 20-10-2015
- Strekking van de antwoorden:

Niet-technische samenvatting:

- We hebben de NTS aangepast aan de gestelde criteria door deze beter te laten aansluiten bij de fundamentele insteek van het project. De herziene NTS bevat ook minder speculatie over de mogelijk translationele waarde. Daarnaast is alles uitgelegd met eenvoudiger taalgebruik.
- 3.4: We hebben alle verwijzingen naar de milde aard van de schokken verwijderd uit de NTS. We bedoelden hier aanvankelijk mee te zeggen dat de elektrische schokken dusdanig van aard zijn dat ze als onprettig (stressvol) worden ervaren, zonder verdere weefselschade te veroorzaken. We zijn het echter met de commissie eens dat in psychologisch opzicht de schokken niet mild zijn en hebben deze toevoeging dan ook verwijderd uit de beschrijving.
- 3.5: Het cumulatieve ongerief voor de dieren is matig of ernstig, afhankelijk van het specifieke experiment. We hebben deze informatie toegevoegd aan de herziene NTS.

Project Proposal:

- We verontschuldigen ons voor deze ongelukkige vergissing. De spelfout is gecorrigeerd in de gehele herziene aanvraag.
- 3.1: We zijn het met de commissie eens dat er inderdaad nog maar weinig werk gepubliceerd is over het voorgestelde PTSS-protocol en dat het woord 'well-established' misschien iets te krachtig is. Desalniettemin zijn er verschillende studies uitgevoerd die de waarde van het voorgestelde PTSS-model bevestigen, waarvan verscheidene door de hoofdonderzoeker zelf (ook in een ander laboratorium). Zowel het fenotype van de PTSS-achtige dieren in het gedrag als de afwijkingen in stress hormoon secretie (die onafhankelijk zijn van de categorisatie als PTSS-vatbaar of -resistent, maar ook waargenomen worden in PTSS patiënten) zijn inmiddels meerdere keren (onafhankelijk) gerepliceerd. Daarnaast zijn andere onderzoekers in het veld dusdanig enthousiast geraakt dat ze het model ook zijn gaan gebruiken en ons benaderd hebben voor eventuele samenwerking ([redacted]). We zijn er dan ook van overtuigd dat dit model het beste diermodel voor PTSS is dat we kunnen gebruiken. We hebben de frasering van 'well-established' echter aangepast en verder beargumenteerd waarom we juist dit PTSS-model willen gebruiken (onderdeel 3.1 van het herziene projectvoorstel).

- 3.1: We verontschuldigen ons voor de onduidelijkheden omtrent de onderzoeksvragen en de gebrekkige achtergrond van het project. We hebben deze sectie nu substantieel uitgebreid. De onderzoeksvragen zijn in principe onafhankelijk van elkaar, al worden ze op een soortgelijke wijze (d.w.z. met hetzelfde PTSS-model) onderzocht. Wij gaan er bij dit onderzoek vanuit dat van alle dieren die we blootstellen aan het trauma, een gedeelte volledig herstelt, en een gedeelte PTSS-achtige verschijnselen ontwikkelt (dit is in vorig onderzoek met het PTSS-model aangetoond). We zijn geïnteresseerd in de verschillen tussen deze twee groepen, en willen die onderzoeken op 3 tijdspunten. Voor onderzoeksvraag 1 gaan we op zoek naar vatbaarheidsfactoren (voorafgaand aan de blootstelling aan trauma) die de latere ontwikkeling van PTSS na een trauma kunnen voorspellen. Zo zouden uiteindelijk individuen gescreend kunnen worden op deze factoren en vatbare personen kunnen worde geëxcludeerd van risicovol werk (bijv. bij de brandweer, politie, of in het leger). Met onderzoeksvraag 2 richten we ons op kenmerken van PTSS-vatbare individuen tijdens de reactie op het trauma zelf (d.w.z. in de tijd rond de traumatische gebeurtenis). Zo zouden vatbare individuen meteen behandeld kunnen worden als ze zich in het ziekenhuis melden, om zo meteen in te grijpen en de ontwikkeling van PTSS te voorkomen. Voor onderzoeksvraag 3 richten we ons op de kenmerken van PTSS-vatbare individuen na het veronderstelde herstel van de traumatische gebeurtenis. We gaan er hierbij vanuit dat de PTSS-resistente dieren op dit tijdstip volledig hersteld zijn van het trauma, maar de PTSS-vatbare dieren nog niet (ze vertonen immers gedragsafwijkingen). In dit stadium heeft dus één groep PTSS ontwikkeld en de andere niet. Door de hersenen van deze groepen te vergelijken verkrijgen we inzicht in de pathologie van PTSS, en zo dus meer informatie over mogelijk targets voor behandeling van de aandoening. We hebben de achtergrondinformatie in onderdeel 3.1 uitgebreid om voldoende informatie te bieden voor onderdeel 3.2. Daarnaast hebben we een extra figuur (Figuur 1) opgenomen in het voorstel ter verduidelijking van de drie onderzoeksvragen.
- 3.1: We erkennen het aangestipte verschil tussen mannen en vrouwen in gevoeligheid voor PTSS. Daar staat echter tegenover dat hoewel het percentage vrouwen dat t.g.v. een trauma PTSS ontwikkelt hoger ligt, het percentage vrouwen dat daadwerkelijk een traumatische gebeurtenis meemaakt in het leven zo'n 30% lager ligt dan bij mannen (Kessler et al. 1995). Daarnaast is het type trauma's dat vrouwen meemaken (bijv. seksueel misbruik, verwaarlozing, verkrachting, etc.) van dusdanig andere aard als bij mannen (die vaker worden blootgesteld aan (niet-sexueel) geweld, rampen, verwondingen, ongelukken en getuige zijn van dood/verwondingen), dat deze trauma's vaker in PTSS resulteren (Olff et al. 2007; Santiago et al. 2013). Bovendien maken deze trauma's gewoonlijk op een jongere leeftijd mee, waarop ze vatbaarder zijn voor PTSS, en gaan ze samen met een sterkere perceptie van bedreiging en verlies van controle over de situatie (Olff et al. 2007). Er zijn studies die suggereren dat als voor deze factoren gecorrigeerd wordt en beide sexen aan soortgelijke traumatische gebeurtenissen blootgesteld worden, vrouwen en mannen een gelijke kans hebben op het ontwikkelen van PTSS (bijv. Maguen et al. 2012). De voornaamste redenen waarom wij mannelijke dieren gebruiken zijn A) omdat het voorgestelde PTSS-inductie protocol alleen gevalideerd is in mannetjes muizen, en B) om het aantal benodigde dieren zo veel mogelijk te beperken. Vanuit humaan onderzoek is namelijk bekend dat de stressgevoeligheid van vrouwen afhankelijk is van de precieze fase in hun menstruele cyclus (ze zijn gevoeliger in de premenstruele fase (Ossewaarde et al. 2010; Lustyk et al. 2012)) en

dat hun brein in deze fasen ook anders reageert op stress en streshormonen (Ossewaarde et al. 2010). Om voor deze factoren te controleren en de bevindingen te kunnen generaliseren naar het gehele geslacht, zou daarom ten eerste de cyclus van de muizen bijgehouden moeten worden, en daarnaast 3x zoveel muizen getest moeten worden om alle punten in de cyclus te meten. Hoewel wij de vertaling van onze bevindingen naar vrouwen erg belangrijk vinden, en dit zeker in de toekomst van plan zijn te gaan testen, lijkt het ons verstandiger voor deze eerste studie te focussen op de groep met de meest stabiele stress respons, waarin het voorgestelde model reeds gevalideerd is (i.e., mannetjes). We hebben deze onderbouwing nu opgenomen in het herziene projectvoorstel (onderdeel 3.1).

- 3.1: We hebben Figuur 1 toegevoegd om meer informatie te verschaffen over de veronderstelde fysiologie en onderliggende neurale circuits van PTSS. We verwachten met name verschillen in het functioneren van het zgn. salience (emotie) netwerk en het cognitieve controle netwerk. Coping styles zijn gerelateerd aan de functie van deze netwerken, maar vrij lastig te meten in de muizen. De mate van freezing tijdens de blootstelling aan de trauma en trigger hebben we al eerder gekwantificeerd, maar bleek helaas niet informatief over de latere ontwikkeling van een PTSS-achtig fenotype in de muizen.
- 3.1: We hebben deze wijziging doorgevoerd en de verwijzing naar de financiering van het project verplaatst naar onderdeel 3.3.
- 3.2: We hebben deze wijzigingen doorgevoerd en de secties verplaatst naar de juiste onderdelen.
- 3.3: We hebben het wetenschappelijk belang van dit onderzoek verder benadrukt in de herziene versie van onderdeel 3.3. Ons doel is om biomarkers te identificeren die de gevoeligheid voor PTSS voorspellen. Dit zouden biomarkers in het brein kunnen zijn, bijv. toegenomen of afgenomen activiteit in een specifiek hersengebied, maar ook in het bloed (bijv. corticosteron niveaus). We werken samen met een andere onderzoeksafdeling die een risicogroep voor de ontwikkeling van PTSS (politieagenten) onderzoekt, en we willen onze biomarkers als eerste in deze populatie testen. Uiteraard kunnen niet exact dezelfde metingen in het humane brein worden gedaan, maar metingen van hersenactiviteit middels fMRI zijn mogelijk, en bloedwaardes zijn vrij eenvoudig vertaalbaar. Daarnaast zou men ook kunnen denken aan genetische tests, naar variatie in genen waarvan we vinden dat ze betrokken zijn bij de vatbaarheid voor PTSS (door te bepalen welke eiwitten de hersencellen met afwijkende activiteit/plasticiteit kenmerken). We hebben de onderbouwing van potentiële translationele waarde van het onderzoeksproject verder uitgebreid in sectie 3.3.
- 3.4.1: In het projectvoorstel worden 3 onderzoeksvragen (1-3) beschreven die zich allemaal op een andere tijdsperiode in de ontwikkeling van PTSS richten (zie ook het antwoord op de tweede vraag over sectie 3.1). Daarmee zijn deze onderzoeksvragen conceptueel volledig onafhankelijk van elkaar, en zijn de resultaten van onderzoeksvraag 1 niet informatief over het design of de haalbaarheid van experimenten 2 en 3. Immers, of er kenmerken zijn tijdens baseline (onderzoeksvraag 1) die latere ontwikkeling van PTSS voorspellen, staat los van mogelijke kenmerken tijdens de blootstelling aan trauma (onderzoeksvraag 2), en van de kenmerken van de uiteindelijke pathologie die zich ontwikkelt na mogelijk tekortschietend herstel (onderzoeksvraag 3).

Elke onderzoeksvraag bestaat echter uit 3 onderdelen/niveaus (A-C), die wel onderling afhankelijk van elkaar zijn. Hierin zijn wel duidelijke go/no go momenten opgebouwd; alleen

wanneer onderdeel A duidelijke aanwijzingen geeft over het precieze type hersencel die zich afwijkend gedraagt, kan onderdeel B uitgevoerd worden, en alleen als er circuits in onderdeel B geïdentificeerd zijn, kunnen deze worden gemanipuleerd in onderdeel C. We hebben de onafhankelijkheid van de onderzoeksvragen verder benadrukt in onderdeel 3.4.1 van het herziene projectvoorstel, en de afhankelijkheid van onderdelen A-C (en de go/no go momenten hierin) verder uitgewerkt door het gehele voorstel. Daarnaast is figuur 2 verplaatst naar sectie 3.4.1, om daar al meer duidelijkheid te scheppen over de opzet van het project.
- 3.4: We hebben een tijdslijn aan onderdeel 3.4.2. van het herziene voorstel toegevoegd (Figuur 3), waar we zeer globaal de tijdslijn van de verschillende experimenten aangeven.
- 3.4.2: De milestones die in onderdeel 3.4.3. beschreven staan gaan niet over de 3 verschillende onderzoeksvragen, maar over de 3 onderdelen (A-C) die ze beslaan. De onderdelen A-C zullen na elkaar onderzocht worden en alleen als het eerdere onderdeel succesvol is gebleken wordt met het volgende onderdeel gestart. We hebben onderdeel 3.4.3. aangepast om dit te verduidelijken.
- 3.4.2: De neurobiologie van het hersenweefsel (of meer specifiek; van de gelabelde cellen die afwijkende activiteit/plasticiteit vertonen) zal worden onderzocht in in-situ hybridisatie en immunohistochemie experimenten. Q-PCR zal op het hersenweefsel van de dieren gebruikt voor onderdeel A helaas niet mogelijk zijn aangezien alle breintjes perfusie-fixatie zullen ondergaan, zodat we hersenplakjes kunnen snijden die we vervolgens onder de microscoop zullen bestuderen voor de identificatie van de afwijkende hersencellen (daar zijn alle dieren voor nodig). Er zullen verschillende sets hersenplakjes worden gesneden, en de resterende sets zullen worden gebruikt voor de in-situ hybridisatie en immunohistochemie experimenten. We hebben deze nu informatie toegevoegd aan onderdeel 3.4.2.
- 3.4.2: Onderdeel B kan alleen uitgevoerd worden als een celpopulatie (en hersengebied) bekend is waarop we ons kunnen richten voor de identificatie van mogelijk afwijkende circuits. We achten het zeer onwaarschijnlijk dat er veranderingen op circuitniveau plaatsvinden zonder bijbehorende veranderingen in neuronale activiteit of plasticiteit. Echter, het nadeel van de voorgestelde transgene muislijnen (cFos-CreERT2 and Arc-CreERT2 mice) is dat de labeling een ‘alles-of-niets’ kleuring inhoudt, d.w.z. zodra het immediate early gene (c-Fos of Arc) aangemaakt wordt, verkleurt de cel. De methode stelt ons dus in staat afwijkende activiteit/plasticiteit te detecteren als het om verschillende aantallen cellen gaat, maar is ongevoelig voor eventuele gradaties in activiteit/plasticiteit (het niveau van c-Fos of Arc-expressie) per cel. Het zou dus eventueel mogelijk zijn dat een identiek aantal cellen activiteit/plasticiteit vertoont, maar in een verschillende mate. In dat geval zullen we in onderdeel A geen verschillen vinden, maar is het toch interessant om onderdeel B uit te voeren. Het is dan echter zaak deze cellen op een andere manier te identificeren, zodat we weten van welke cellen we het circuit moeten bepalen in onderdeel B. In dit geval zullen we een amendement indienen om onderdeel A opnieuw uit te voeren, maar waarbij we de breintjes invriezen na het offeren van de dieren om vervolgens Q-PCR experimenten uit te kunnen voeren op potentieel interessante hersengebieden. Wanneer deze experimenten dan verschillen laten zien (in c-Fos en Arc-expressie, maar bijvoorbeeld ook in andere immediate early genes of stress-gerelateerde genen) tussen de resistente en PTSS-vatbare dieren, kan het corresponderende gebied (of celtype) getarget worden voor onderdeel B.

- 3.4.2: De optische vezels die in het brein geïmplanteerd worden zijn slechts enkele mm's lang en zullen slechts minimaal uit de schedel van de muizen steken. Er zal ook geen fotostimulatie plaatsvinden tijdens de gedragstesten. Daarmee zullen de geïmplanteerde vezels dus geen invloed hebben op de bewegingsvrijheid van de muizen tijdens de gedragstesten.
-3.4.3: We hebben de criteria voor beslispunt A en B (de go/no go momenten) verder toegespitst in onderdeel 3.4.3.

Description of Animal Procedures:
- De ongeriefscore van de muizen die een PTSS-achtig fenotype ontwikkelen was reeds ingeschat als ernstig. Dit in tegenstelling tot vorige DEC-aanvragen (Ru DEC 2014-175 en Ru DEC 2014-243), waarin in het ongerief ingeschaald was op matig-ernstig. We zijn ons ervan bewust dat de herhaaldelijke blootstelling aan de elektrische schokken in een verschillende context (zoals gebeurt voor de PTSS-inductie) een ernstige mate van ongerief voor de PTSS-vatbare dieren kan opleveren aangezien het consequenties heeft op de lange termijn. We willen de commissie er echter op wijzen dat vergelijkbare elektrische schokken vaker worden toegepast in conditionerings experimenten (fear conditioning), en dat de verlichting (~100 lux) en het lawaai (~70 dB), slechts van matige intensiteit zijn, en daardoor naar verwachting slechts als mild aversief ervaren worden.
- Hoewel we meegaan met de commissie in de beoordeling van het ongerief van de dieren die PTSS-achtige verschijnselen vertonen als ernstig, leert onze ervaring met deze PTSS-inductie methode ons dat de resistente dieren zeker geen ernstig ongerief van de PTSS inductie ervaren. In eerder ingediende DEC-aanvragen (Ru DEC 2014-175 en Ru DEC 2014-243) is aan het directe ongerief van de blootstelling aan de elektrische schokken in deze groep een lagere ongeriefscore van matig ongerief toegekend, gebaseerd op de waarneming dat de meeste dieren goed herstellen van de PTSS-inductie methode en geen duidelijk fenotype ontwikkelen ten gevolge ervan. Het is belangrijk te weten dat de muizen geen enkele vorm van blijvend lichamelijk ongerief van de procedure ondervinden, en het psychisch ongerief is slechts beperkt tot de PTSS-achtige dieren. Ogenschijnlijk zien alle muizen er dan ook gezond uit, en is er niets bijzonders aan hun op te merken. Wanneer we deze ingreep (die overeenkomt met de procedures die normaliter worden toegepast tijdens fear conditioning experimenten; waaraan een matig ongerief wordt toegekend) vergeleken wordt met de impact van bijvoorbeeld operaties is deze naar onze mening zelfs verwaarloosbaar. We vragen de commissie dan ook om haar oordeel over de ongeriefscore te herzien na het verkrijgen van deze extra informatie.
- DAP1 vraag A1: We willen beginnen met het onderzoeken van de reactie op de combinatie van de trauma en trigger blootstelling zoals beschreven staat in onderzoeksvraag 2a1. De (neurale) reactie op beide gebeurtenissen zal de meest robuuste labeling veroorzaken van geactiveerde/plastische hersencellen. Echter, een dergelijk robuuste reactie en labeling tijdens beide gebeurtenissen geeft ook meer achtergrondsignaal, dat mogelijk niet relevant is voor onze vergelijking van de PTSS-vatbare en resistente dieren en veel 'ruis' veroorzaakt. Vorig onderzoek heeft aangetoond dat de trigger absoluut noodzakelijk is voor de ontwikkeling van PTSS-achtige symptomen in de dieren (Lebow et al. 2012); wanneer ze niet 'getriggerd' worden, herstellen de dieren prima. Men merkt dit ook gedragsmatig aan de muizen; na de trigger gedragen ze zich veel gestresster dan na het trauma, terwijl ze bij deze

laatste aan meer en intensievere elektrische schokken zijn blootgesteld. Dit zou enerzijds kunnen betekenen dat er een cruciaal proces plaatsvindt tijdens de trigger wat specifiek is voor PTSS-achtige dieren. Een dergelijk proces kan het best geadresseerd kan worden door selectief te kijken naar de neurale respons op de trigger. Anderzijds zou het ook zo kunnen zijn dat er een proces in gang wordt gezet tijdens de blootstelling aan het trauma, dat er vervolgens voor zorgt dat de trigger de muizen over de drempel duwt. We willen daarom graag beide opties apart van elkaar onderzoeken, maar alleen in onderdeel A van het onderzoek. Gebaseerd op de bevindingen in onderdeel A zullen we ons toespitsen of 1 van de 3 methoden om de afwijkende trauma-gerelateerde activiteit/plasticiteit het best te targeten. We hebben deze toelichting nu ook opgenomen in de herziene beschrijving van de experimenten.

- DAP1 vraag A2, IV: We zien de beschreven bepaling van de neuroendocriene functie als zeer waardevolle toevoeging aan ons onderzoek. Eerder onderzoek met dit model voor PTSS (Lebow et al. 2012) heeft namelijk aangetoond dat de PTSS-vatbare dieren een onderdrukte corticosteron stress respons vertonen t.o.v. de resistente dieren; een bepaling die compleet onafhankelijk van de categorisatie van de dieren als PTSS-vatbaar of resistent (gebaseerd op gedrag) plaatsvindt, en in overeenstemming is met het neuroendocrience fenotype van patiënten die lijden aan PTSS (Yehuda 2001). Daarnaast hebben we deze bevindingen in ons eigen lab gerepliceerd. Een groot vraagstuk in het humane PTSS onderzoek is wanneer deze afwijkende neuroendocrience functie zich precies ontwikkelt; vormt deze een vatbaarheidsfactor voor PTSS, of is deze het gevolg van de ontwikkeling van de ziekte? Er is een opkomende theorie dat corticosteron noodzakelijk is voor adequaat herstel van stress (Hermans et al. 2014), en daarmee dus erg belangrijk bij de mogelijke ontwikkeling van PTSS. In dit onderzoek meten we allereerst de basale corticosteron niveaus in de ochtend en avond om het circadiane ritme te meten, aangezien hierin ook afwijkingen zijn geconstateerd in PTSS-patiënten (Daskalakis et al. 2013). Daarnaast zullen we de corticosteron reactie op het trauma en de trigger meten om te bepalen of PTSS-vatbare en resistente dieren in hun reactie verschillen, zoals gesuggereerd wordt in sommig humaan onderzoek (McFarlane 2000; Witteveen et al. 2010). Mocht dit zo zijn dan zou toediening van hydrocortison na trauma blootstelling een mogelijke interventie methode zijn voor PTSS (Schelling et al. 1999, 2001, 2002; Zohar et al. 2011). Als laatste meten we dus de reactie op een gestandaardiseerde stressor in het lab, waarbij al eerder verschillen tussen de vatbare en resistente dieren zijn gerapporteerd. Door deze metingen te verrichten in dezelfde dieren als waarin we de hersenactiviteit/plasticiteit bepalen, kunnen we vervolgens correlationele analyses verrichten naar de relatie tussen deze bepalingen, om zo een beter idee te krijgen van de onderliggende neurale mechanismen aan deze neuroendocriene afwijkingen.
- DAP1 vraag A2. De immunohistochemische en in situ-hybridisatie experimenten zijn aan de beschrijving toegevoegd. Het is echter niet ons primaire doel met deze experimenten de activatie of plasticiteit van de gelabelde cellen te bevestigen. We willen middels deze experimenten extra informatie verkrijgen over het type cel dat gelabeld is (excitatoir vs inhibitoir) en de (stress-gerelateerde) eiwitten die deze cellen tot expressie brengen. Zoals eerder aangegeven is Q-PCR helaas niet mogelijk met dit weefsel.
- DAP1 vraag A3: Voor onderzoeksvraag 2 (het bepalen van abnormale activiteit/plasticiteit van hersencellen tijdens de blootstelling aan het trauma en de trigger) is het belangrijk te weten welke hersenactiviteit/plasticiteit direct gerelateerd is aan de traumatische ervaring

en niet aan overige omstandigheden, zoals de injectie van 4-OHT of de blootstelling aan een nieuwe omgeving. In tegenstelling tot tamoxifen (dat een delay kent in de labeling van enkele uren) labelt 4-OHT namelijk vrijwel direct alle actieve/plastische cellen, en veroorzaakt daarmee mogelijk een soort achtergrondruis. De controle dieren zijn in deze experimenten nodig ter vergelijking met de getraumatiseerde groepen, om zo de neuronale respons op het PTSS-inductie protocol te kunnen identificeren. Daarnaast biedt de vergelijking van de controle groep met de resistente muizen informatie over een adaptieve respons op trauma's, wat ook erg waardevolle informatie is.

- DAP1 vraag D2: We danken de commissie voor deze kritische noot en zullen de dieren dagelijks checken voor een goede bepaling van humane eindpunten. We hebben dit gewijzigd in het projectvoorstel. Daarnaast hebben we de vermelding van groepshuisvesting verwijderd in dit onderdeel.
- DAP1 vraag J: We hebben deze wijzigingen doorgevoerd.
- DAP1 vraag K: Het cumulatieve ongerief voor de dieren is matig of ernstig (afhankelijk van de groep waartoe het dier behoort). We hebben deze informatie toegevoegd aan de herziene beschrijving van de dierprocedures.

NB. We hebben de benodigde aanpassingen voor de dierprocedures van onderdeel A ook doorgevoerd in de onderdelen B en C, zoals de commissie suggereerde.

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- De antwoorden hebben geleid tot aanpassing van de aanvraag.
- Datum: 09-11-2015
- Strekking van de vragen:

Niet-technische samenvatting:

-De NTS bevat nog spelfouten, het taalgebruik is nog niet overal vereenvoudigd, en de formulering is soms wat moeizaam. De samenvatting is nu een stuk langer dan 500 woorden geworden.

-3.5 De percentages dieren die respectievelijk mild, matig of ernstig ongerief ondervinden zijn nog niet gegeven. Ook klopt het totale aantal dieren bij 3.5 niet met het aantal opgegeven bij 3.3. De onderzoekers geven een uitgebreide beschrijving van de oorzaken van het ongerief, maar kunnen hier volstaan met het vermelden van het cumulatieve ongerief en de percentages dieren die het respectievelijk betreft.

Project Proposal:

-3.2 De haalbaarheid van de doelstelling is nog niet onderbouwd.

Description of Animal Procedures:

-De inschatting van het ongerief als gevolg van de onvermijdbare en onvoorspelbare elektrische schokken om PTSS te induceren bij een deel van de dieren is in strijd met de richtlijn waarnaar in de Wet op de Dierproeven wordt verwezen. Volgens deze richtlijn is alleen de onvermijdbaarheid van de schokken al reden om het ongerief als ernstig te beschouwen. Bovendien is de commissie van mening dat de gehanteerde verlichting en het lawaai voor muizen niet van slechts matige intensiteit zijn. De aanname dat de dieren die geen PTSS oplopen als gevolg van de schokken, derhalve die schokken ook als minder onaangenaam hebben ervaren dan de dieren die wel PTSS hebben opgelopen, is bovendien nergens op gebaseerd. De commissie zal in haar advies voor de CCD uiteenzetten waarom zij op dit punt afwijkt van de ongeriefclassificatie door de onderzoekers, tenzij de onderzoekers alsnog deze ongeriefclassificatie aanpassen.

-DAP1, vraag A2. De functie van corticosteron als herstelhormoon na stress is wetenschappelijk aangetoond. De onderzoekers worden verzocht hiervoor een relevante internationale referentie toe te voegen en niet alleen naar PTSS-werk te verwijzen.

-De commissie geeft u ter overweging om meteen al extra muizen aan te vragen om Q-PCR te kunnen doen. Een veel gehanteerde volgorde is om eerst met Q-PCR te onderzoeken of het gen kwantitatief reageert, en daarna pas in situ hybridisatie en immunohistochemie toe te passen.

- Datum antwoord: 11-11-2015
- Strekking van de antwoorden:

Niet-technische samenvatting:

- We hebben de NTS verbeterd en ingekort.

- 3.5: We hebben bij punt 3.5 de percentages dieren die matig/ernstig ongerief ondervinden toegevoegd. Ook komen nu de aantallen dieren opgegeven bij punten 3.5 en 3.3 met elkaar overeen.

Project Proposal:

- 3.2: We hebben deze onderbouwing nu toegevoegd. Er staat nu: "[redacted] supporting its relevance, innovative nature, and feasibility. We have the experience and facilities in-house to perform the required studies. Researchers involved in this proposal have extensive experience with the proposed PTSS model, as well as the use of the proposed techniques (qPCR, immunohistochemistry, neuronal tracing, and optogenetics) for the experiments. Moreover, in collaboration with others, the researchers have already performed (successful) preliminary tests in the proposed transgenic mouse lines. The use of an available light-sheet microscope will further facilitate the analyses of fluorescent labeling."

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Description of Animal Procedures:

- We hebben de ongeriefclassificatie van alle dieren die blootgesteld worden aan de onvermijdbare elektrische schokken op aanraden van de DEC aangepast naar ernstig.
- DAP1, vraag A2: Het idee dat corticosteron als herstelhormoon na stress functioneert (ook in het brein) vindt inderdaad een steeds breder draagvlak in de internationale wetenschappelijke gemeenschap. We hebben daarom in overeenstemming met dit commentaar enkele referenties naar ander relevant wetenschappelijk onderzoek toegevoegd.
- We danken de commissie voor deze suggestie en zijn het met haar eens dat qPCR experimenten ons extra inzicht zullen verschaffen in de mechanismen die aan de veranderde activiteit/plasticiteit van de geïdentificeerde cellen ten grondslag liggen. Daarnaast zullen ze ons ook extra informatie opleveren over de aard van de cellen. We hebben deze experimenten nu toegevoegd in de herziene projectaanvraag bij onderdeel A. We beschrijven nu dat we na de identificatie van de relevante hersengebieden (in de eerste groep dieren beschreven in onderdeel A) in een nieuwe groep dieren eerst een qPCR zullen verrichten op de geïdentificeerde fluorescente cellen (door ze eerst te isoleren d.m.v. 'Fluorescence-Activated Cell Sorting' (FACS)) om een idee te krijgen welke (activiteit/plasticiteit- en stress-gerelateerde) genen ten grondslag liggen aan de veranderde activiteit/plasticiteit. Vervolgens zullen we door middel van in situ hybridisatie en immunohistochemie (in het hersenweefsel verkregen van de eerste groep dieren) bevestigen dat deze genen tot expressie worden gebracht in de fluorescent gekleurde cellen.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

9. Eventuele adviezen door experts (niet lid van de DEC)
   - Aard expertise
   - Deskundigheid expert
   - Datum verzoek
   - Strekking van het verzoek
   - Datum expert advies
   - Expert advies

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig.
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.

C. Beoordeling (inhoud):

1. Het project is:
   - uit wetenschappelijk oogpunt verantwoord
2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.
3. De DEC onderschrijft het belang van de doelstelling, namelijk 'to elucidate aberrant neuronal circuit function differentiating the PTSD-vulnerable from [redacted] bain to enhance the understanding of PTSD etiology.' De te behalen onderzoeksresultaten zullen duidelijk maken of er verschillen zijn te identificeren in het functioneren van hersencircuits tussen muizen die wel of niet PTSS ontwikkelen na blootstelling aan een combinatie van een traumatische gebeurtenis en een daaropvolgende trigger. Er wordt onderzocht of er verschillen zijn te identificeren voorafgaand, tijdens, of na de PTSS-inductie. Deze resultaten zullen leiden tot een beter begrip van de ziekte, en kunnen op termijn leiden tot betere preventie en behandeling van PTSS. Deze aandoening kent een hoge prevalentie in de bevolking, en heeft een grote impact op de kwaliteit van leven van patiënten en hun naasten. Maatschappelijk is dit onderzoek van belang, omdat de resultaten kunnen bijdragen aan de ontwikkeling van effectievere therapieën voor, en mogelijk ook preventie van, PTSS, wat zou resulteren in gezondheidswinst voor veel mensen.
4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Deze groep heeft veel ervaring in dit onderzoeksveld en met de voorgestelde dierproeven, zoals ook tot uiting is gekomen in [redacted]. De gekozen aanpak leidt tot betrouwbare uitspraken over de verschillen in het functioneren van hersencircuits tussen muizen die gevoelig dan wel resistent zijn voor het ontwikkelen van PTSS, en geeft meer inzicht in de moleculaire mechanismen die hierbij betrokken zijn.
5. Er is geen sprake van bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren.
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Het ongerief wordt hoofdzakelijk bepaald door de inductie van PTSS. De DEC schat het ongerief als gevolg van de benodigde gedragstesten, de bloedafnames en de i.p. injectie in als licht. Het ongerief als gevolg van de stress door de bewegingsbeperking schat de DEC in als matig. De commissie is van mening dat alle dieren die worden blootgesteld aan de onverwachte en onvermijdbare elektrische schokken ernstig ongerief ondergaan, ongeacht het feit of zij later PTSS zullen ontwikkelen of niet. De DEC is van mening dat de combinatie van al deze factoren tot ernstig ongerief leidt. Het cumulatief ongerief voor de muizen in de beschreven vergunningaanvraag is dus juist ingeschat als ernstig voor alle dieren. Indien voor de uitvoering van een deel van de proeven PTSS-inductie niet nodig blijkt, zal het ongerief voor 4% van de dieren matig in plaats van ernstig zijn.
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.** De doelstelling van het project kan niet gerealiseerd worden zonder proefdieren of door gebruik van minder complexe diersoorten. Een aandoening die ontstaat door de verwerking

in de hersenen van een traumatische gebeurtenis en die effecten op het gedrag heeft kan niet goed bestudeerd worden zonder proefdiermodellen.

8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de **vermindering** van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat en proportioneel ten opzichte van de gekozen strategie en de looptijd. De DEC is het eens met het beschreven onderzoeksmodel en de onderbouwing van het aantal benodigde dieren. Door het toepassen van meerdere go/no go momenten wordt onnodig gebruik van proefdieren voorkomen. De DEC is van oordeel dat het project kan worden uitgevoerd met maximaal 1992 muizen.
9. Het project is in overeenstemming met de vereiste van de **verfijning** van dierproeven. De experimentele handelingen bij de dieren zullen worden uitgevoerd door hierin getrainde onderzoekers, waardoor de stress voor de dieren zoveel mogelijk wordt beperkt. Dagelijkse controles van de dieren zorgen ervoor dat bij onverwacht optredend ongerief tijdig kan worden ingegrepen. Waar mogelijk worden transgene dieren gebruikt waardoor PTSS-inductie niet nodig is en het ongerief voor de dieren beperkt wordt tot matig. De DEC is ervan overtuigd dat de dierproeven zo humaan mogelijk worden uitgevoerd.
   Er is geen sprake van belangwekkende milieueffecten.
10. De niet-technische samenvatting is een evenwichtige weergave van het project, zelfstandig leesbaar, beknopt en begrijpelijk geformuleerd.

D. Ethische afweging

Op basis van de onder C genoemde overwegingen komt de DEC tot de volgende ethische afweging.

Met dit onderzoek worden belangrijke wetenschappelijke inzichten verworven in de etiologie van PTSS bij muizen, en in de moleculaire mechanismen die hierbij betrokken zijn. De resultaten zullen duidelijk maken of er verschillen zijn te identificeren in het functioneren van hersencircuits tussen muizen die wel PTSS ontwikkelen en muizen die geen PTSS ontwikkelen na blootstelling aan een combinatie van een traumatische gebeurtenis en een daaropvolgende trigger. Het is aannemelijk dat deze inzichten op termijn zullen bijdragen aan effectievere therapie en mogelijk ook preventie van PTSS bij mensen. Het belang van meer inzicht in de etiologie van PTSS en de ontwikkeling van nieuwe interventies acht de DEC substantieel, gezien de hoge prevalentie van PTSS in de bevolking en de impact daarvan op de kwaliteit van leven.

Tegenover dit substantiële belang staat het gegeven dat (vrijwel) alle dieren ernstig ongerief zullen ondervinden als gevolg van de PTSS-inductie in combinatie met de daarvoor benodigde handelingen. De commissie is zich er van bewust dat het gebruikte model zeer belastend is voor de dieren. Dit vergt een sterke rechtvaardiging in de vorm van een doelstelling die haalbaar geacht mag worden en die minimaal van substantieel belang is. Verder dient duidelijk te zijn dat alle mogelijkheden voor vervanging, vermindering en/of verfijning ook daadwerkelijk toegepast zullen worden. De commissie is er van overtuigd dat dit het geval is. Het gebruik van de dieren en het daarbij optredende ongerief is onvermijdelijk, wil men de doelstelling kunnen realiseren. Het realiseren van die doelstelling, meer inzicht in de etiologie van PTSS, kan een belangrijke bijdrage leveren aan het ontwikkelen van nieuwe interventies voor de behandeling en preventie van PTSS.

De DEC is daarom van oordeel dat het hier boven geschetste belang de onvermijdelijke nadelige gevolgen van dit onderzoek voor de dieren, in de vorm van angst, pijn of stress, rechtvaardigt. Aan de

eis dat het belang van de experimenten op dient te wegen tegen het ongerief dat de dieren wordt berokkend, is voldaan.

E. Advies

1. Advies aan de CCD

- De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
    - Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.

2. Het uitgebrachte advies is gebaseerd op consensus.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Radboud Universiteit Nijmegen
Stichting Katholieke Universiteit Nijmegen Instantie voor Dierenwelzijn
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0900 28 000 28 (10 ct/min)
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Onze referentie
Aanvraagnummer
AVD103002015327

Bijlagen
2

Datum 26 november 2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte [redacted],
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 25 november 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002015327. Gebruik dit nummer wanneer u contact met de CCD opneemt.

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

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

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10300
Naam instelling of organisatie:	Radboud Universiteit Nijmegen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	41055629
Straat en huisnummer:	Geert Grooteplein 10
Postbus:	9101, [redacted]
Postcode en plaats:	6500 HB NIJMEGEN
IBAN:	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer:	UMC St Radboud

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

Gegevens plaatsvervangende verantwoordelijke onderzoeker

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

Gegevens verantwoordelijke uitvoering proces

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

Gegevens gemachtigde

BSN:	[redacted]
Naam:	[redacted]
Postbus:	9101
Postcode en plaats:	6500 HB NIJMEGEN (628)
Wilt u een nieuwe machtiging afgeven?	Nee
Wat mag de gemachtigde doen?	[ ] Een projectvergunning aanvragen [ ] Een wijziging op een verleende projectvergunning aanvragen [ ] Een melding doorgeven op een verleende projectvergunning [ ] Een bezwaarschrift indienen en daarover communiceren met de Centrale Commissie Dierproeven en alle andere handelingen verrichten die nodig zijn voor een goede afwikkeling van het bezwaarschrift [x] Alle bovenstaande opties

Over uw aanvraag

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

Over uw project

Geplande startdatum:	25 december 2015
Geplande einddatum:	25 december 2020
Titel project:	Neural correlates of post-traumatic stress disorder: natural resilience as key for interven
Titel niet-technische samenvatting:	Onderzoek naar de hersenkenmerken die beschermen tegen post-traumatische stress st
Naam DEC:	RU DEC
Postadres DEC:	Postbus 9101, 6500 HB Nijmegen ([redacted])
E-mailadres DEC:	[redacted]

Betaalgegevens

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

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] Melding Machtiging [x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Nijmegen
Datum:	25 november 2015

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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Stichting Katholieke Universiteit Nijmegen Instantie voor Dierenwelzijn
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Onze referentie
Aanvraagnummer
AVD103002015327

Bijlagen
2

Datum 26 november 2015
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 26 november 2015
Vervaldatum: 26 december 2015
Factuurnummer: 15700327
Ordernummer: 040823-461220/2015-090/[redacted]

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD103002015327	€ 741,00

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

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD103002015327
Bijlagen
1

Datum 11 JAN 2016
Betreft Beslissing Aanvraag projectvergunning Dierproeven

Geachte [redacted]
Op 25 november 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neural correlates of post-traumatic stress disorder: natural resilience as key for intervention" met aanvraagnummer AVD103002015327. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. U kunt met uw project "Neural correlates of post-traumatic stress disorder: natural resilience as key for intervention" starten. De vergunning wordt afgegeven van 11 januari 2016 tot en met 25 december 2020.
Overige wettelijke bepalingen blijven van kracht.

Beoordeling achteraf
Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1, lid 1d en lid 3, in de wet. Meer informatie over de eisen bij een beoordeling achteraf vindt u in de bijlage.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie RU DEC gevoegd. Dit advies is opgesteld op 24 november 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. In aanvulling daarvan is een algemene voorwaarde vanuit artikel 10, lid 1a van de wet opgenomen.

Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

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

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

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

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

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

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze,

[redacted]

ir. G. de Peuter
Algemeen Secretaris

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

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Stichting Katholieke Universiteit Nijmegen
Adres:	Postbus 9101, [redacted]
Postcode en plaats:	6500 HB NIJMEGEN
Deelnemersnummer:	10300

deze projectvergunning voor het tijdvak 11 januari 2016 tot en met 25 december 2020, voor het project "Neural correlates of post-traumatic stress disorder: natural resilience as key for intervention" met aanvraagnummer AVD103002015327, volgens advies van Dierexperimentencommissie RU DEC.
De functie van de verantwoordelijk onderzoeker is [redacted] Voor de uitvoering van het project is Instantie voor Dierenwelzijn verantwoordelijk.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 25 november 2015
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 25 november 2015;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 25 november 2015;
c Advies van dierexperimentencommissie d.d. 24 november 2015, ontvangen op 25 november 2015.

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
A. Identification of PTSD-associated neuronal activation and plasticity	Muizen (Mus musculus) / cFos- CreERT2 and Arc-CreERT2; volwassenen	1104	Ernstig / severe	Cumulatief ernstig ongerief.
B. Characterization of PTSD-associated neuronal circuits	Muizen (Mus musculus) / cFos- CreERT2 and Arc-CreERT2; volwassenen	312	Ernstig / severe	Indien er voor Approach II wordt gekozen zullen maximaal 72 dieren worden gebruikt, en het ongerief zal cumulatief matig zijn, in plaats van cumulatief ernstig voor Approach I.
C. Manipulation of PTSD-associated neuronal circuits	Muizen (Mus musculus) / cFos-CreERT2 en Arc-CreERT2; volwassenen	576	Ernstig / severe	Cumulatief ernstig ongerief.

Voorwaarden

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

In artikel 10, lid 1a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in overleg met de Instantie voor Dierenwelzijn te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Voorschriften

In verband met het ernstige ongerief wordt er een beoordeling achteraf bij dit project vereist. Deze moet binnen een jaar na de afloop van de vergunning plaatsvinden, uiterlijk 24 december 2020.

Weergave wet- en regelgeving

Dit project en wijzigingen

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

Verzorging

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

Pijnbestrijding en verdoving

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

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

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

Beoordeling achteraf
Volgens artikel 10a1, lid 1d en lid 3 van de wet worden projecten waarbij niet-menselijke primaten worden gebruikt, projecten die als ernstig ingedeelde dierproeven omvatten of een dierproef die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, achteraf beoordeeld worden. In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.
Deze beoordeling zal uiterlijk december 2021 plaatsvinden. Er zal dan beoordeeld worden of de

doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst van lijden van de proevendieren conform de vergunning waren.