Centrale Commissie Dierproeven

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.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of neem telefonisch contact op. (0900-2800028).

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 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 1.3

☐ Wijziging > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.1

☐ Melding > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.2

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

Naam instelling of organisatie	Stichting Katholieke Universiteit Nijmegen			
Titel, voorletters en achternaam van de portefeuillehouder	Titel Prof. dr.	Voorletters H.	Achternaam Van Krieken	☐ Dhr. ☐ Mw
E-mailadres contactpersoon				
Titel, voorletters en achternaam van de diens gemachtigde (indien van toepassing)	Titel	Voorletters [redacted]	Achternaam [redacted]	☐ Dhr. ☐ Mw
E-mailadres gemachtigde	instantievoordierenwelzijn@radboudumc.nl			

Vul de gegevens van het postadres in.

Straat en huisnummer	Geert Grooteplein		29 / HP231
Postcode en plaats	6525 EZ	Nijmegen	
Postbus, postcode en plaats	9100	6500HB	Nijmegen

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☐ Dhr. ☒ Mw.
Functie	[redacted]	
Afdeling	[redacted]	

		Telefoonnummer	[redacted]
		E-mailadres	[redacted]@radboudumc.nl
1.5	*(Indien van toepassing)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	(Titel) Naam en voorletters	[redacted] ☐ Dhr. ☐ Mw.
		Functie	[redacted]
		Afdeling	[redacted]
		Telefoonnummer	[redacted]
		E-mailadres	[redacted]@radboudumc.nl
1.6	*(Indien van toepassing)* Vul hier de gegevens in van de persoon aan wie de portefeuillehouder de verantwoordelijkheid inzake de algemene uitvoering van het project en de overeenstemming daarvan met de projectvergunning heeft gedelegeerd.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		E-mailadres	
1.7	*(Optioneel)* Vul hier de gegevens in van de Instantie voor Dierenwelzijn	Telefoonnummer	[redacted]
		E-mailadres	instantievoordierenwelzijn@radboudumc.nl
1.8	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		X Nee	

2 Over uw aanvraag

2.1	Gaat uw aanvraag over een *wijziging* op een vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn?	☒ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder kort de wijziging en de onderbouwing daarvan weer. Geef in de originele formulieren (niet-technische samenvatting, projectvoorstel en bijlage dierproeven) duidelijk aan (bij voorbeeld in een andere kleur) waar de projectaanvraag wijzigt. Ga daarna verder met vraag 6.
2.2	Gaat uw aanvraag over een *melding* op een vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn?	☒ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder weer wat deze melding inhoudt en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	01 - 07 - 2022
		Einddatum (t/m)	30 - 06 - 2027
3.2	Wat is de titel van het project?	Neural mechanisms underlying stress hormone effects on memory flexibility	
3.3	Wat is de titel van de niet-technische samenvatting?	De effecten van stresshormonen op geheugenflexibiliteit	
3.4		Naam DEC	RU DEC
		Postadres	Postbus 9101, 6500 HB, Nijmegen (HP 231)

Wat is de naam van de Dierexperimentencommissie (DEC) van voorkeur?	E-mailadres	dierexperimentencommissie@radboudumc.nl

4 Factuurgegevens

4.1 (indien factuuradres afwijkt van de gegevens uit vraag 1.3) Vul de gegevens van het factuuradres in.

Naam: RadboudUMC / [redacted]	Afdeling: [redacted]	
Straat: Geert Grooteplein		Huisnummer: 29 / HP231
Postcode: 6525 EZ	Plaats: Nijmegen	
Postbus: 9101	Postcode: 6500 HB	Plaats: Nijmegen
E-mail: instantievoordierenwelzijn@radboudumc.nl		

4.2 (optioneel) Vul hier het ordernummer van de instelling in.

Ordernummer: [redacted] CDL projectnummer: 2022-0008

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel — Aantal bijlage(n) dierproeven 3

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☐

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD en per post naar de Centrale Commissie Dierproeven (voor adresgegevens zie website)

Ondertekening door de portefeuillehouder namens de instellingsvergunninghouder of gemachtigde (zie 1.8). 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 C 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	IvD
Plaats	Nijmegen
Datum	05 - 05 - 2022
Handtekening	

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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 mechanisms underlying stress hormone effects on memory flexibility

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 of routine production
[] Research into environmental protection in the interest of human or animal health or welfare
[] Research aimed at preserving the species subjected to procedures
[] Higher education or training
[] Forensic enquiries
[] Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.1.

Stress and stress hormones affect memory
Stressful encounters lead to orchestrated signaling by various hormones, peptides and neurotransmitters in both the periphery and the brain. Stress rapidly activates the sympathetic nervous system, which triggers the release of catecholamines such as epinephrine and norepinephrine (NE) from the adrenal medulla and sympathetic nerve endings. NE is also directly released in the brain upon stress exposure by the activation of noradrenergic cells in the locus coeruleus. Stress further induces a more delayed activation of the hypothalamo-pituitary-adrenocortical axis that leads to the release of glucocorticoids (CORT; corticosterone in rodents, cortisol in humans) from the adrenal cortex. Extensive evidence from both animal and human studies indicates that these stress mediators not only prepare an individual for the acute consequences of a dangerous or threatening situation, but also induce long-term effects on learning and memory. Prior work from our laboratory in rodents has established a robust conceptual framework for understanding how NE and CORT act on neural circuits [e.g., amygdala, hippocampus and prefrontal cortex (PFC)] in modulating different memory functions [redacted]. NE and CORT were shown to enhance the consolidation of new memories, but impair the retrieval of previously acquired memories [redacted]. 'Evidence from several studies indicates that interactions between NE and CORT may be essential in regulating memory. For example, an attenuation of NE signaling with a β-adrenoceptor antagonist administered into the amygdala or several other brain regions blocks glucocorticoid effects on both the consolidation and retrieval of memory (1-3). By contrast, an attenuation of glucocorticoid signaling with a glucocorticoid receptor antagonist resulted in that a much higher dose of NE was needed to modulate memory. These findings indicate that CORT modulates these memory functions by having a permissive influence on the noradrenergic system. However, we recently found that the effects of NE and CORT on memory are not always synergistic and that they induce opposite effects on episodic-like accuracy of memory. The amygdala is particularly important for orchestrating these NE and CORT effects by influencing consolidation and retrieval processes in other brain regions such as the hippocampus and PFC (1, 2). A tight regulation of stress hormone effects on memory is usually considered to be highly adaptive and pivotal for survival, but aberrant processing of memories under stress lies at the core of several stress-related mental disorders, including post-traumatic stress disorder (PTSD) and anxiety disorders (4).

Stress effects on memory flexibility
When making decisions, we not only draw on previous experience, but also use information we have not directly experienced by combining knowledge from multiple discrete items or events to infer new relationships. This ability to flexibly infer previously unobserved relationships is critical for adaptive behavior (5) and thought to be dependent on PFC-hippocampal circuits (6, 7). Memory flexibility has been hypothesized to involve chaining together memories for discrete events at the time of choice. Together with prior memories, such higher-order relationships may form a "relational" or "cognitive map" of the world (8). The hippocampus has been attributed to holding a cognitive map (9), and to mediate associations between sequential events (10). In general, it is assumed that inference across events can be supported by pattern separation (11) and/or pattern completion processes (12), which are dependent on different hippocampal subregions. Computational and initial neuroimaging findings in humans suggest that the anterior hippocampus (i.e., dorsal hippocampus in rodents) supports pattern completion, whereas the posterior hippocampus (i.e., ventral hippocampus in rodents) is involved in pattern separation (13). On the other hand, the PFC enables the reactivation and updating of related elements during the decision-making process (6, 14). A similar functional anterior-posterior distinction may exist within the PFC (13). There are no direct monosynaptic projections from the PFC to the hippocampus, and the nucleus reuniens of the ventral midline thalamus is an important anatomical link between both brain regions (15).

Recent findings from human studies demonstrated that stress—beyond the known effects on consolidation and retrieval— also hampers this flexibility of memory (3). For instance, stressed participants who were trained in a virtual navigation task showed an increased reliance on familiar paths and reduced traversal of shortcuts when these became available. These findings dovetail with recent evidence suggesting that stress may interfere with the capacity to flexibly and intentionally control memory retrieval processes (16). Likewise, stress-induced cortisol release shortly before initial learning or pharmacological elevations of noradrenergic activity have been shown to impair participants' ability to generalize across past experiences when required to flexibly transfer memories to novel situations (17, 18). An impaired capacity to adequately and flexibly link memories may result in rigid memories (19, 20), and could explain the overly strong emotional responding to trauma-related cues in PTSD patients and may complicate therapeutic interventions (3, 4). **Although highly relevant both for our understanding of emotional modulation of memory and for clinical contexts, the mechanisms and circuits involved in these stress-induced deficits in memory flexibility are completely unclear.** Thus, a better understanding of the endocrine and neural mechanisms underlying deficits in memory flexibility during stressful situations may lead to new avenues for the treatment of such disorders.

In this project, we will test the hypothesis that NE and CORT, in a synergistic manner, induce deficits in memory flexibility by impairing PFC-hippocampal activity, and that the amygdala plays an important role in orchestrating these stress hormone effects on PFC-hippocampal function. Figure 1 shows a schematic presentation of this hypothesis. We will investigate this in a mouse model of memory flexibility. As of yet, such stress and stress hormone effects on impairing the flexibility of memory have been investigated almost exclusively in humans. However, animal studies are indispensable to provide causal evidence for the neural mechanisms underlying stress hormone effects on memory flexibility, which cannot be done in humans. In all experiments, we will train mice on a multi-day memory inference task consisting of three different stages (14). First, in the 'observational learning' stage, mice will learn a set of associations between auditory and visual cues via mere exposure. Second, in the 'conditioning' stage, the mice will learn that half of the visual cues predict the delivery of a rewarding outcome. After mice have completed the training protocol, we will implement a memory inference test. In this third stage, we will present auditory cues in isolation, without visual cues or outcomes, and measure evidence for inference from the auditory cue to reward outcome.

In order to investigate our hypothesis that stress hormones impair memory flexibility by acting on PFC-hippocampal circuits, we will have three different sets of manipulations:

- We will administer NE and CORT systemically and examine effects on memory flexibility and the neuronal representation of memory flexibility.
- We will administer NE and CORT directly into different loci of the PFC-hippocampal circuit to examine their involvement in regulating memory flexibility.
- We will combine systemic NE and CORT administration and chemogenetic manipulation of these neural circuits to provide causal evidence for the modulation of neural circuit function in the establishment of the effects of these two stress hormones on memory flexibility.

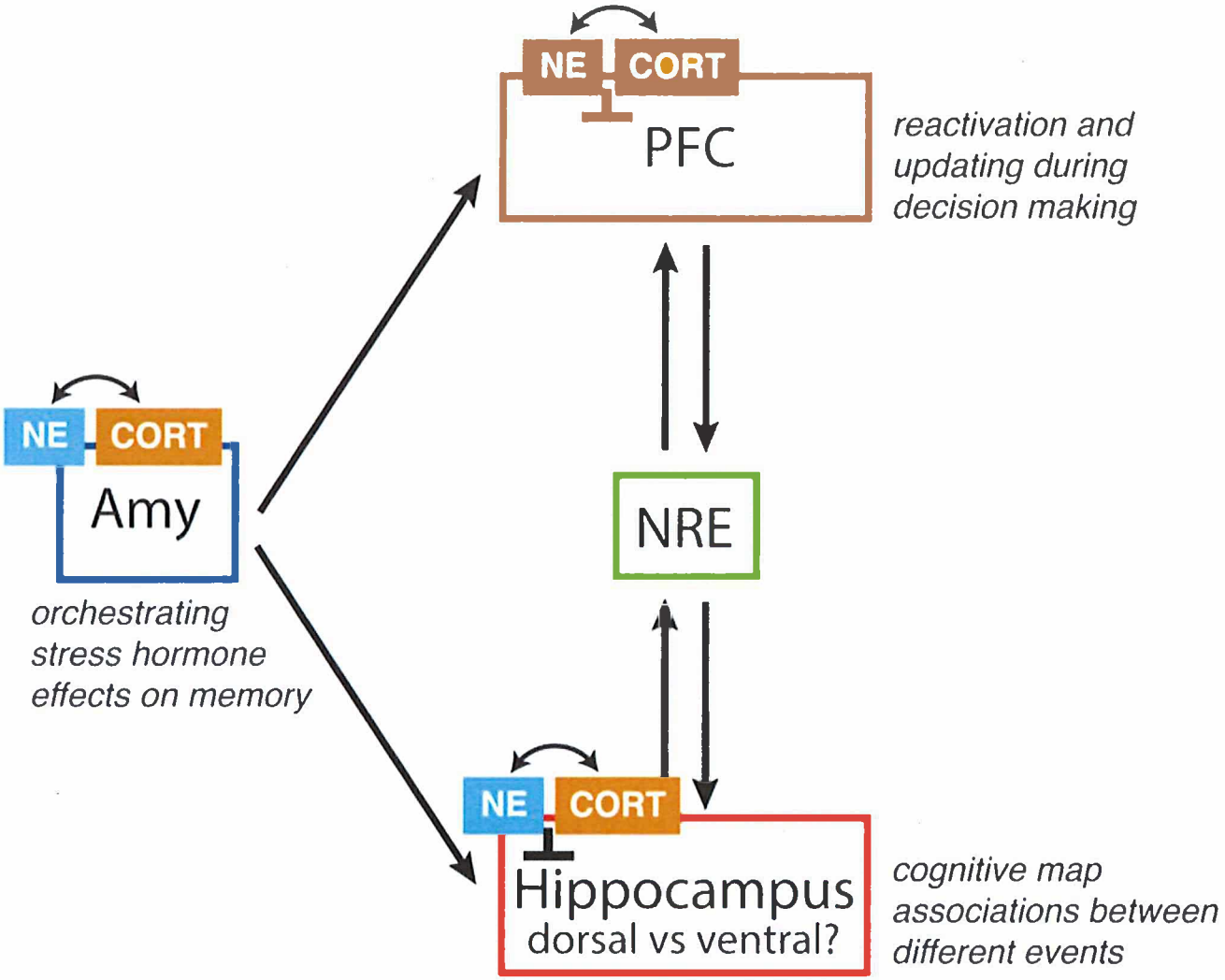


Figure 1: *Schematic representation of the hypothesis how NE and CORT synergistically induce deficits in memory flexibility by impairing PFC-hippocampal activity. The amygdala (Amy) is hypothesized to play an important role in orchestrating stress hormone effects on PFC-hippocampal function. The nucleus reuniens (NRE) is an important anatomical link between the PFC and hippocampus. CORT = corticosterone; PFC = prefrontal cortex; NE = norepinephrine.*

[redacted] D., Schwabe, L., Roozendaal, B. Stress, glucocorticoids and memory: implications for treating fear-related disorders. *Nat Rev Neurosci* ***18,*** 7–19 (2017).
5. Shohamy, D., Adcock, R.A. Dopamine and adaptive memory. *Trends Cogn Sci* ***14,*** 464–472 (2010).
6. Schlichting, M.L., Preston, A.R. Memory integration: neural mechanisms and implications for behavior. *Curr Opin Behav Sci* ***1,*** 1-8 (2015).
7. Preston, A.R., Eichenbaum, H. Interplay of hippocampus and prefrontal cortex in memory. *Curr Biol* ***23,*** R764–R773 (2013).
8. Cohen, N.J., Eichenbaum, H. Memory, Amnesia, and the Hippocampal System (MIT Press) (1993).
9. O'Keefe, J., Nadel, L. The Hippocampus as a Cognitive Map (Clarendon Press) (1978).

10. Fortin, N.J., Agster, K.L., Eichenbaum, H.B. Critical role of the hippocampus in memory for sequences of events. *Nat Neurosci* ***5,*** 458–462 (2002).
11. Preston, A.R., Shrager, Y., Dudukovic, N.M., Gabrieli, J.D. Hippocampal contribution to the novel use of relational information in declarative memory. *Hippocampus* ***14,*** 148-152 (2004).
12. Shohamy, D., Wagner, A.D. Integrating memories in the human brain: hippocampal-midbrain encoding of overlapping events. *Neuron* ***60,*** 378-389 (2008).
13. Schlichting, M.L., Mumford, J.A., Preston, A.R. Learning-related representational changes reveal dissociable integration and separation signatures in the hippocampus and prefrontal cortex. *Nat Commun* ***6,*** 8151 (2015).
14. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183,*** 228-243 (2020).
15. Vertes, R.P., Hoover, W.B., Szigeti-Buck, K., Leranth, C. Nucleus reuniens of the midline thalamus: link between the medial prefrontal cortex and the hippocampus *Brain Res Bull* ***71,*** 601-609 (2007).
16. Quaedflieg, C.W.E.M., Schneider, T.R., Daume, J., Engel, A.K., Schwabe, L. Stress impairs intentional memory control through altered theta oscillations in lateral parietal cortex. *J Neurosci* ***40,*** 7739–7748 (2020).
17. Dandolo, L.C., Schwabe, L. Stress-induced cortisol hampers memory generalization. *Learn Mem* ***23,*** 679–683 (2016).
18. Kluen, L.M., Agorastos, A., Wiedemann, K., and Schwabe, L. Noradrenergic stimulation impairs memory generalization in women. *J Cogn Neurosci* ***29,*** 1279–1291 (2017).
19. Halligan, S.L., Michael, T., Clark, D.M., Ehlers, A. Posttraumatic stress disorder following assault: the role of cognitive processing, trauma memory, and appraisals. *J Consult Clin Psychol* ***71,*** 419-431 (2003).
20. Simon-Kutscher, K., Wanke, N., Hiller, C., Schwabe, L. Fear without context: acute stress modulates the balance of cue-dependent and contextual fear learning. *Psychol Sci* ***30,*** 1123–1135 (2019).

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

• If applicable, describe all subobjectives

Given that several mental disorders are characterized by altered stress response patterns and that stress-induced changes in memory are thought to be a driving force in stress-related mental disorders, the **ultimate goal** of the project is to enhance mechanistic understanding of aberrant forms of memory processing under stress contributing to stress-related mental disorders, with the hope that it will lead to novel treatment approaches.

To achieve our ultimate goal, the project will pursue three **immediate goals** to enhance mechanistic insight into how the stress hormones NE and CORT impact memory flexibility and its underlying modulation of neural circuit function:
1. to determine the effects of systemic administration of NE and CORT on memory flexibility and the neuronal representation of memory flexibility.
2. to determine the effects of NE and CORT, and their functional interaction, in specific brain regions on memory flexibility and the neuronal representation of memory flexibility.
3. to provide causal evidence for the modulation of neural circuit function in the establishment of the effects of NE and CORT on memory flexibility.

3.2.2 Provide a justification for the project's feasibility.

We have >30 years of experience in investigating the neural mechanisms underlying stress and stress hormone effects on memory (with review papers in some of the highest-ranking journals in the field (e.g. Nature Reviews Neuroscience [redacted] and carrying out behavioral procedures, pharmacological and neural circuit manipulations in rodents. By using animal models of learning and memory, we have shown that NE and CORT enhance the formation and stabilization of new memory traces, whereas they disturb the recall of older memories. In close collaboration with clinical researchers, we have successfully translated our animal findings to new glucocorticoid-based therapy to improve the treatment of PTSD and anxiety disorders [redacted] We have the expertise, equipment, and all facilities in-house to perform the required studies (mouse behavior, stress hormone administration, viral injection for neural circuit manipulation, immunohistochemistry, et cetera). The animal studies are aligned with parallel studies in healthy human subjects [redacted] to directly facilitate translation. In addition, the project will be carried out at [redacted] in Nijmegen, a worldwide recognized institute for Neuroscience research. [redacted] provides a high quality scientific, experimental, and administrative support to its scientists ensuring the successful outcome of research projects.

[redacted]

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

[X] No

[] Yes > Describe which laws and regulations apply and describe the effect on the welfare of the animals and the feasibility of the project.

3.3 Relevance

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

Stress has a major impact on memory, driven by the concerted action of various stress mediators on the brain (1). This is primarily a highly adaptive phenomenon as it allows the individual to prepare for similar future events and thereby enable more efficient coping. However, compelling evidence indicates that aberrant processing of stressful experiences lies at the core of several stress-related mental disorders, including PTSD and phobias (2). Currently, 7.7 million people in Europe alone suffer from PTSD, and many millions more suffer from anxiety disorders. Treatment is still seriously inadequate; currently less than half of PTSD patients currently benefit from treatment. A hallmark characteristic of PTSD is a deficit in memory flexibility which may result in rigid memories that lack contextual details (3, 4). Such rigid memories could explain the overly strong emotional responding to trauma-related cues (e.g., odors and tones) in PTSD patients and may complicate therapeutic interventions. There is further recent evidence that directly links an impairment of the flexible control of memory retrieval, as observed under acute stress (5), to PTSD symptoms (6). **Therefore, it is vital that we better understand how stress and stress hormones not only impact the consolidation and retrieval of memory, but also memory flexibility.** The proposed studies in mice (and parallel studies in humans) will substantially influence basic science in the area of memory and contribute to a better understanding of the effects of stress mediators on the brain and memory, and possibly help in the future to develop better treatment for those who suffer from (non-adaptive) emotional memories.

1. McGaugh, J.L. Memory--a century of consolidation. *Science* ***287,*** 248–251 (2000).
2. Green, B. Post-traumatic stress disorder: symptom profiles in men and women. *Curr. Med. Res. Opin.* ***19,*** 200–204 (2003).
3. Wirz, L., Bogdanov, M., Schwabe, L. Habits under stress: mechanistic insights across different types of learning. *Curr Opin Behav Sci* ***20,*** 9–16 (2018).
4. Simon-Kutscher, K., Wanke, N., Hiller, C., Schwabe, L. Fear without context: acute stress modulates the balance of cue-dependent and contextual fear learning. *Psychol Sci* ***30,*** 1123–1135 (2019).
5. Quaedflieg, C.W.E.M., Schneider, T.R., Daume, J., Engel, A.K., Schwabe, L. Stress impairs intentional memory control through altered theta oscillations in lateral parietal cortex. *J Neurosci* ***40,*** 7739–7748 (2020).
6. Mary, A., et al. Resilience after trauma: the role of memory suppression. *Science* ***367,*** 8477 (2020).

3.3.2 Who are the project's stakeholders? Describe their specific interests.

The main stakeholders for this project are the neuroscientists that will carry out the work, other basic neuroscientists with similar research interests, the experimental animals, and the patients (in the future).

The **neuroscientists** that will work on the project will benefit as this work will lead to several publications that they will author. This in turn will advance their careers.

Other basic neuroscientists with an interest in cognitive neuroscience may also benefit from this research as they could use the behavioral task and theoretical model in their own lab. Also, the findings of the articles will benefit the larger neuroscience community as the obtained information aids to the available knowledge in this field.

The **cognitive psychologists** that will work on the human studies will also benefit as this work is expected to generate hypotheses that will be tested further in humans.

The **animals** that will serve as experimental subjects in the project are also stakeholders as they will experience discomfort via their participation in the experiments (through brain surgery, single housing and food restriction needed for the behavioral task).

Finally, **patients** with a stress-related mental disorder may be the ultimate stakeholders as the project in the future may lead to the development of better treatment for (non-adaptive) memories.

3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the diferent phases in the project, the coherence, the milestones, selection points and decision criteria.
In this project, we aim to elucidate the cognitive, endocrine and neural mechanisms through which stress impairs memory flexibility. To achieve this goal, we will have three aims:

In DAP1, we will examine aim 1 of the project proposal: to determine the effects of systemic administration of NE and CORT on memory flexibility and the neuronal representation of memory flexibility. We will train mice on a multi-day memory inference task consisting of three different stages (1). Figure 2 shows a schematic representation of the general design of the task. First, in the 'observational learning' stage (lasting 3-6 days), mice will learn a set of associations between auditory and visual cues via mere exposure. On each trial, an auditory and visual cue will be presented serially and contiguously: auditory cue followed by associated visual cue. Second, in the 'conditioning' stage (lasting 4-5 days), subjects will learn that half of the visual cues predict the delivery of a rewarding outcome ('set 1'), while the other half predict the delivery of a neutral outcome ('set 2'). On each trial, a visual cue and outcome will be presented serially and contiguously: visual cue followed by outcome delivery. Rewarding outcomes will be drops of sucrose and neutral outcomes drops of water.

After mice will have completed the training protocol (observational learning and conditioning stages), we will implement a memory inference test (stage 3, lasting 1 day). Shortly before the memory inference test, mice will be given a systemic administration of three different doses of yohimbine (a noradrenergic stimulant), CORT or their respective vehicles. Yohimbine is a selective alpha2-adrenoceptor antagonist that increases NE levels both peripherally and in the brain (2, 3) and CORT is the main endogenous glucocorticoid in rodents that is known to bind to both mineralocorticoid and glucocorticoid receptors in the brain (4). Earlier experiments have shown that the most effective dose of yohimbine/NE and CORT is always found within a restricted dose range but varies depending on the specific memory component (e.g. memory consolidation vs memory retrieval) that is investigated, the intrinsic stressfulness of the memory task and the brain regions involved (2). We have not yet determined the dose-response relationship of yohimbine or CORT on impairing memory flexibility in the memory inference task. Three different dosages of yohimbine and CORT is the absolute minimum for us to determine the complex dose-response relationship (2). To control for possible stress effects of the injection procedure itself on behavior or neuronal activation, additional control groups are added that do not receive an injection. On the memory inference test, we will present auditory cues from set 1 and set 2 in isolation, and measure evidence for inference from the auditory cue to outcome by quantifying reward-seeking behavior, defined as the time spent in the outcome area in the 20 s period after the offset of the auditory cues (see Figure 2). Control mice (treated with the same doses of yohimbine or CORT) will be presented visual cues from set 1 and set 2 in isolation to verify that the stress hormone administration does not induce general effects on the animals' incentive to seek the reward or affect the retrieval of the learned association (visual cue-outcome). The animals will be killed by perfusion fixation after the memory inference test for later immunohistochemistry experiments. Neuronal activity readouts will be acquired by measuring different immediate early gene (IEG) expression responses in relevant brain regions (e.g., PFC, hippocampus, and amygdala). These experiments will thus determine whether systemic administration of NE and CORT impair memory flexibility and how this is associated with changes in neuronal activity. The neural activity patterns will aid in determining the choice of target brain regions for aim 2 and 3.

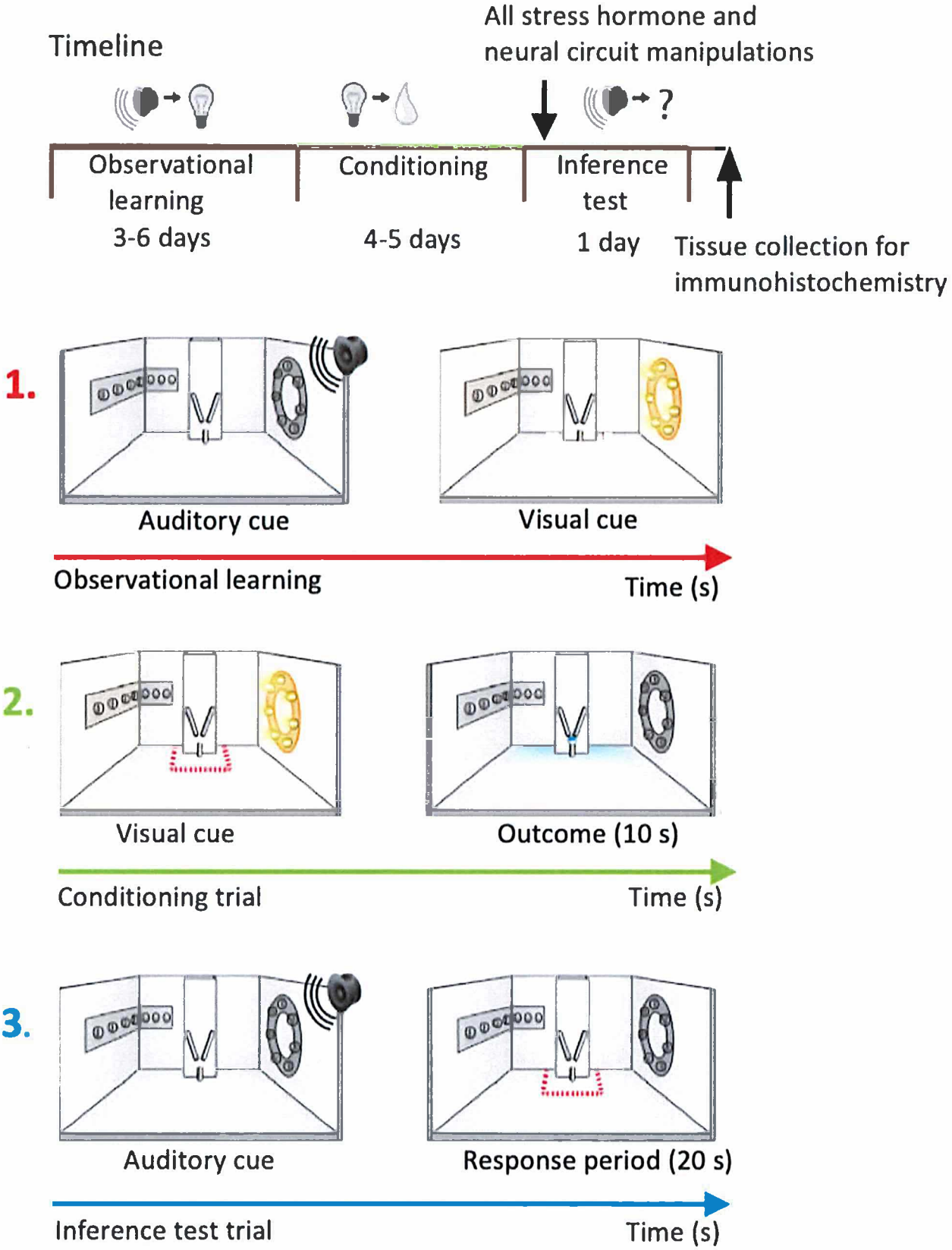


Figure 2: *General design of the memory inference task with the different stages explained. Top: timeline for the task. For all aims, stress hormone and neural circuit manipulations will take place prior to the inference test (stage 3). 1. Example observational learning trial. An auditory cue, from either set 1 or 2, is followed by*

presentation of the associated visual cue. 2. Example conditioning trial. A visual cue and outcome will be presented serially and contiguously. Half of the visual cues predict the delivery of a rewarding outcome ('set 1'), while the other half predict the delivery of a neutral outcome ('set 2'). Rewarding outcomes will be drops of sucrose and neutral outcomes drops of water. Red dotted line delineates the outcome area around the dispenser. 3. Example inference test trial. An auditory cue from set 1 and set 2 will be presented in isolation. Evidence for inference from the auditory cue to outcome will be assessed by quantifying the time spent in the outcome area in the 20 s period after the offset of the auditory cues. Adapted from Barron et al (1).

In DAP2, we will examine aim 2 of the project proposal: to determine the effects of NE and CORT, and their functional interaction, in specific brain regions on memory flexibility and the neuronal representation of memory flexibility. All mice will undergo stereotaxic surgery for implantation of bilateral guide cannulas into relevant brain regions. Based on our hypothesis (Figure 1) and current knowledge from mainly human neuroimaging studies, the dorsal hippocampus, ventral hippocampus, PFC and amygdala are prime candidates (1, 5, 6), but a final selection will be made after we obtain the neuronal activity findings of DAP1. After the postsurgical recovery period, they will be trained on the memory inference task. The optimal settings of the task will be determined in DAP1. Shortly before the memory inference test (stage 3), different doses of NE or CORT will be administered via the implanted cannula and we will examine how this will affect memory inference. We will also investigate whether NE and CORT interact in influencing memory inference by a combined administration of NE and a glucocorticoid antagonist or of CORT and a noradrenergic antagonist. Here, we note that if clear negative results are found in DAP1 (the absence of any stress hormone effect on memory flexibility), then DAP2 (and DAP3) will no longer be carried out (go/no-go decision). The procedure of the memory inference test is the same as described above for DAP1. All mice will be killed by perfusion fixation after the memory inference test for immunohistochemical analysis of IEG expression responses in the brain and to check for correct cannula placement. The neuronal activity measures will provide insights into how local stress hormone administration into these brain regions influences neuronal activity in other brain regions and thus affect functional connectivity during the memory inference test.

In DAP3, we will examine aim 3 of the project proposal: to provide causal evidence for the modulation of neural circuit function in the establishment of the effects of NE and CORT on memory flexibility. For this, we will combine DREADD (designer receptor exclusively activated by designer drugs) technology to selectively activate or silence different sites within the PFC and hippocampus with systemic stress hormone manipulations. A first experiment will test the hypothesis that NE and CORT impair memory flexibility by suppressing activity of the PFC and hippocampus (see project proposal, section 3.1, for a schematic representation of this hypothesis). Mice will be injected intracranially with an adeno-associated virus encoding the respective DREADD receptor (e.g., hM4D, hM3D, or control), that will allow us to selectively silence or activate different sites of the PFC-hippocampal circuit. By using both excitatory and inhibitory DREADD manipulations, we can determine whether an activation of these PFC and hippocampal sites blocks the impairing effects of NE and CORT on memory flexibility whereas a silencing of these brain sites mimics the impairing effects of NE and CORT can thereby causes an occlusion of the stress hormone effects on memory flexibility. A second experiment will test the hypothesis that NE and CORT impair memory flexibility by reducing functional connectivity between the PFC and hippocampus. As there are no direct monosynaptic projections from the PFC to the hippocampus, we will use two different approaches to manipulate PFC-hippocampal functional connectivity. In Experiment 2a, mice will be injected unilaterally with an adeno-associated virus encoding the respective DREADD receptors into the PFC and a second injection into either the contralateral or ipsilateral hippocampus. The asymmetrical manipulation (i.e., contralateral injection into the PFC and hippocampus) will affect PFC-hippocampal connectivity in both hemispheres, whereas the symmetrical manipulation (i.e., ipsilateral injection into the PFC and hippocampus) will remain PFC-hippocampal connectivity in one hemisphere intact and thus serves as control. In Experiment 2b, we will inject the adeno-associated virus encoding the respective DREADD receptors bilaterally into the nucleus reuniens, which is an important anatomical link between the PFC and hippocampus. In a third experiment, we will test the hypothesis that amygdala projections to the PFC and hippocampus are critically involved in mediating the effects of NE and CORT on memory flexibility. To target these specific amygdala projections, a retrograde adeno-associated virus expressing Cre recombinase will be injected into the PFC or hippocampus, while an inhibitory or excitatory Cre-dependent adeno-associated virus coding the respective DREADD receptor (e.g., hM4D, hM3D, or control) will be injected into the amygdala. For all experiments, the animals will be trained on the memory inference task, as explained for aim 1. Shortly before the memory inference test (stage 3), mice will receive systemic administration of a low dose of the cognate ligand clozapine to activate the DREADD receptors as well as a single optimal dose of yohimbine, CORT or their respective vehicles. All mice will be killed by perfusion fixation after the memory inference test for later immunohistochemistry experiments and to ensure specific viral targeting placement. Thereby, this series of experiments will provide causal and mechanistic evidence for the critical recruitment for PFC-hippocampal circuits in mediating the effects of NE and CORT on memory flexibility, and how the amygdala plays a critical role in orchestrating these stress hormone effects on PFC-hippocampal function.

Our approach is to perform the experiments necessary to answer our research questions in a structured and (largely) sequential manner. Figure 3 shows a schematic representation of the coherence of the different components and different steps of the project. The three DAPs complement each other and performing those experiments will allow us to thoroughly characterize the effect of stress hormone administration on memory flexibility, as well as their neural underpinnings. DAP1 (aim1) will examine the dose-dependent effect of

systemic NE and CORT administration on memory inference, as well as their neural underpinnings. DAP1 will further determine the optimal settings for the memory inference task. DAP2 and DAP3 aim to further characterize and manipulate the underlying circuitry. In DAP2 (aim2) we will administer stress hormones locally into specific brain regions and examine effects of these two stress hormones (as well as functional interactions between them) on memory flexibility and on changes in neuronal activity in other brain regions. In DAP3 (aim3) we will experimentally manipulate these identified pathways to provide causal and mechanistic evidence for the modulation of neural circuit function in the establishment of the effects of NE and CORT on memory flexibility. Each DAP will provide information that will inform subsequent DAPs (summarized in Figure 3). We have "selection points" for each DAP which we describe as being based on the results of other aims. This especially refers to practical matters, such as behavioral task settings, drug dosages, and areas to target for drug manipulations. In this sense, each aim provides information that will inform that decision. These "selection points" also provide an opportunity for reflection where we could decide that some (sub)experiments no longer need to be performed. Here, we note that if findings from DAP1 show absolutely negative results (i.e. no stress hormone effects), then we will not perform the experiments described in DAP2 and DAP3 (go/no-go decision). Further, if we observe a large difference in memory inference between non-injected and saline/vehicle-injected animals and we are unable to reduce this difference by adjusting the handling sessions or injection method, this will also be considered a no-go criterium. However, we also want to highlight that, in case of inconclusive findings in DAP1, DAP2 and DAP3 serve as complementary experiments by investigating each mechanism/aspect with greater specificity and depth, and thus can resolve this inconclusiveness (e.g. inconclusive data on the effects of systemic administration of stress hormones in DAP1 might be clarified by conducting experiments using local drug administration into specific brain regions as described in DAP2).

Of note, it is unlikely that we will perform all experimental conditions described in the different DAPs We cannot a priori determine exactly which experiments will or will not be necessary as their necessity depends on the results of earlier DAPs. Nevertheless, due to the high potential value of the results, we find it essential to describe and include all possible conditions here, and evaluate their necessity after performing the experiments of earlier DAPs (selection point).

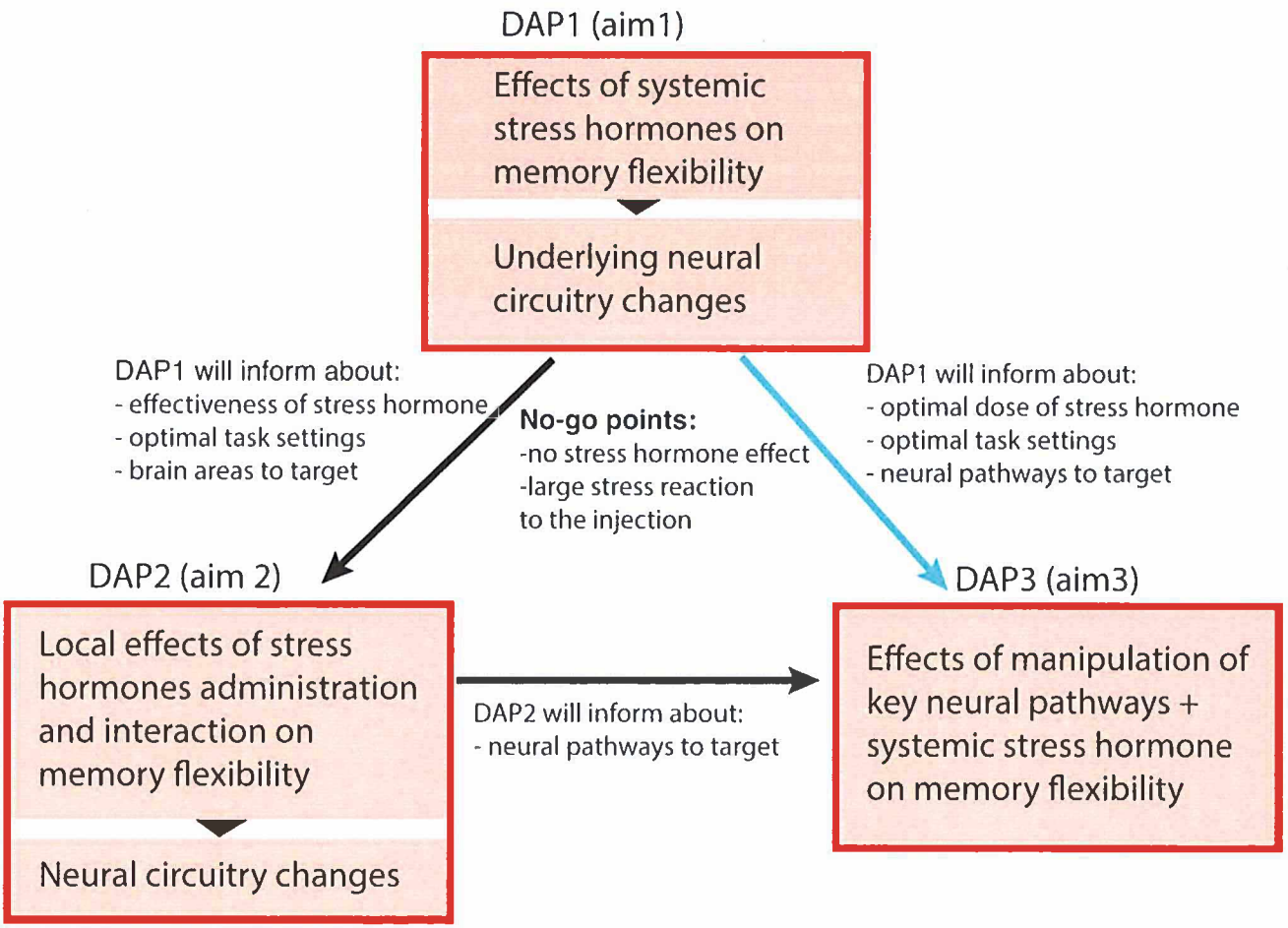


***Figure 3**: Schematic representation of the coherence of the different components and different steps of the project. Black arrows: sequence of experiments and main information transfer; Blue arrow: additional information transfer. The three DAPs complement each other and performing those experiments will allow us to thoroughly characterize the effect of stress hormone administration on memory flexibility, as well as their*

neural underpinnings. DAP1 (aim1) will examine the dose-dependent effect of systemic NE and CORT administration on memory inference, as well as their neural underpinnings. DAP 1 will further determine the optimal settings for the memory inference task. DAP2 and DAP3 aim to further characterize and manipulate the underlying circuitry. In DAP2 (aim2) we will administer stress hormones locally into specific brain regions and examine effects of these two stress hormones (as well as functional interactions between them) on memory flexibility and on changes in neuronal activity in other brain regions. In DAP3 (aim3) we will experimentally manipulate these identified pathways to provide causal and mechanistic evidence for the modulation of neural circuit function in the establishment of the effects of NE and CORT on memory flexibility.

1. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183,*** 228-243 (2020).
2. Szemeredi, K. et al., Simultaneous measurement of plasma and brain extracellular fluid concentrations of catechols after yohimbine administration in rats. Brain Research 542, 8-14 (1991).
3. https://inchem.org/documents/pims/pharm/yohimbin.htm
4. Reul, J. M., de Kloet, E. R. Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology 117, 2505-2511 (1985).
5. Roozendaal, B., McGaugh, J.L. Memory Modulation. Behavioral Neuroscience 125, 797–824 (2011).
6. Preston, A.R., Eichenbaum, H. Interplay of hippocampus and prefrontal cortex in memory. Curr Biol 23, R764–R773 (2013).

3.4.2 Provide a justification for the strategy described above.

The three aims serve as a systematic approach to assess whether our main hypothesis holds true. It is important to emphasize, however, that we have taken measures to ensure that valuable data can be extracted from our experiments, regardless of whether the data fit our hypothesis or not. Our strategy to answer our research questions in a structured and (largely) sequential manner, i.e., first systemic stress hormone administration, followed by local stress hormone administration, and finally DREADD technology to provide causal evidence, we have successfully used in previous projects aimed at investigating the effect of stress hormones on other memory processes and underlying neural mechanisms [redacted]. The different experimental techniques have proven to be highly effective in elucidating stress hormone effects on memory in the rodent brain. We built in all necessary control conditions (e.g., non-injection control groups and visual control cues during the memory inference test), such that we are able to draw specific conclusions. Thereby, this project will produce output parameters covering a wide spectrum; ranging from behavioral parameters, to neuronal networks, to underlying mechanisms. These data will be combined and compared across experiments, to - in the end - sketch a coherent picture of the effects of stress hormone administration on brain mechanisms underlying flexibility of memory. As a consequence, the aims are not only complementary in their conceptual framework and rationale, but also on practical details - each aim provides information that will substantiate decisions and experimental settings of later aims.

3.4.3 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	Determining stress hormone effects on memory flexibility
2	Determining the effect of local administration of stress hormones into specific brain areas on memory flexibility
3	Providing causal evidence for the modulation of neural systems in regulating stress hormone effects on memory flexibility

Centrale Commissie Dierproeven

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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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 serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number 1	Type of animal procedure Determining stress hormone effects on memory flexibility

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
In this part of the project, we will examine aim 1 of the project proposal: to determine the effects of systemic administration of norepinephrine (NE) and corticosterone (CORT) on memory flexibility and the neuronal representation of memory flexibility.

We will train and test mice on a multi-day memory inference task consisting of three different stages (see project proposal, section 3.4.1, for a schematic representation of the general design of the task). First, in the 'observational learning' stage, mice will learn a set of associations between auditory and visual cues via mere exposure. On each trial, an auditory and visual cue will be presented serially and contiguously: auditory cue followed by associated visual cue. Second, in the 'conditioning' stage, subjects will learn that half of the visual cues predict the delivery of a rewarding outcome ('set 1'), while the other half predict the delivery of a neutral outcome ('set 2'). On each trial, a visual cue and outcome will be presented serially and contiguously: visual cue followed by outcome delivery. Rewarding outcomes will be drops of sucrose and neutral outcomes drops of water.

After mice will have completed the training protocol (observational learning and conditioning stages), we will implement a memory inference test (stage 3). Shortly before the memory inference test, mice will be given a single systemic administration of yohimbine (a noradrenergic stimulant), CORT or their respective vehicles (saline for yohimbine, 5% ethanol in saline for CORT). Yohimbine is a selective alpha2-adrenoceptor antagonist that increases NE levels both peripherally and in the brain and CORT is the main endogenous glucocorticoid in rodents that is known to bind to both mineralocorticoid and glucocorticoid receptors in the brain. Earlier experiments have shown that the most effective dose of yohimbine/NE and CORT is always found within a restricted dose range but varies depending on the specific memory component (e.g. memory consolidation vs memory retrieval) that is investigated, the intrinsic stressfulness of the memory task and the brain regions involved. We have not yet determined the dose-response relationship of yohimbine or CORT on impairing memory flexibility in the memory inference task. Given the complex dose-response effects of both NE and CORT (moderate doses have an effect on memory whereas lower or higher doses are ineffective), testing three different dosages is the absolute minimum for us to determine this dose-response relationship. To control for possible stress effects of the injection procedure itself on behavior or neuronal activation, additional control groups are added that do not receive an injection. On the memory inference test, we will present auditory cues from set 1 and set 2 in isolation, without visual cues or outcomes. We will measure evidence for inference from the auditory cue to outcome by quantifying reward-seeking behavior, defined as the time spent in the outcome area in the 20 s period after the offset of the auditory cues. A reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2. The reward-seeking bias is the primary outcome parameter for the behavioral measure of memory inference. In other groups of mice (treated with the same doses of yohimbine or CORT), we will present visual cues from set 1 and 2 in isolation, without auditory cues or outcomes, and quantify a reward-seeking bias. These control groups are necessary to verify that the stress hormone administration selectively affects memory inference and does not induce general effects on the animals' incentive to seek the reward or affect the retrieval of the learned association (visual cue-outcome).

The animals will be killed by perfusion fixation 30-90 min after the inference test for later immunohistochemistry experiments. Neuronal activity readouts will be acquired by measuring different immediate early gene (IEG) expression responses in different brain regions (e.g., hippocampus, prefrontal cortex and amygdala) after the memory inference test and stress hormone administration. Figure 1 shows the timeline and general design of the experiments on this DAP.

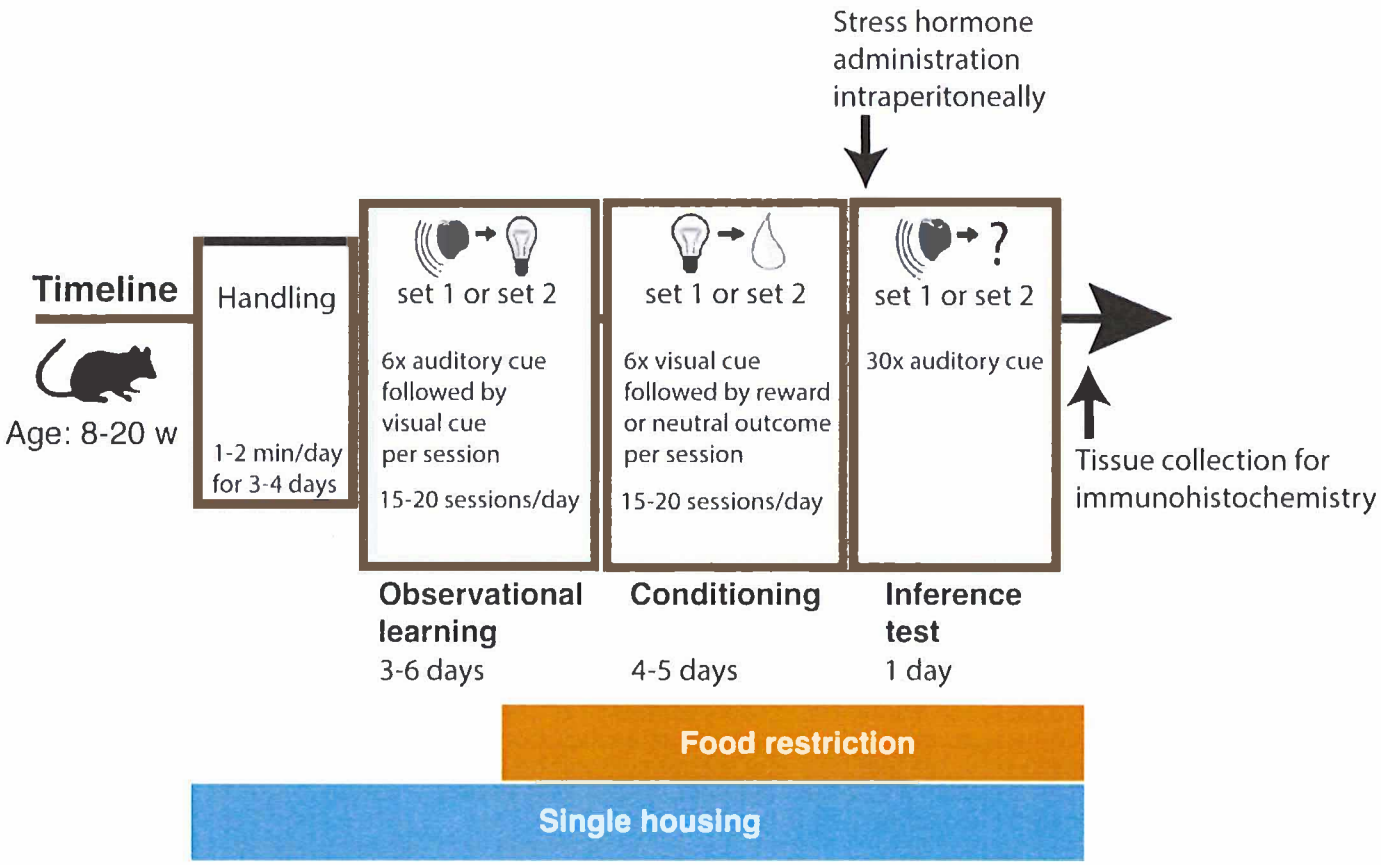


Figure 1: *Timeline and general design of the experiments on this DAP.*

Primary outcome parameters
- Behavioral measure of memory inference: The reward-seeking bias in response to the auditory cues quantified as the difference in reward-seeking behavior for set 1 and set 2.
- Neuronal activity measure: Immunohistochemistry is used to measure the IEG expression in brain after the memory inference test and stress hormone administration.

Justification
Firstly, the behavioral measure of memory inference will allow us to test whether the two stress hormones have similar or differential effects on memory inference. The presentation of auditory cues and visual cues (in different groups of animals) during the memory inference test is required to verify that the stress hormone administration specifically impacts memory inference (i.e., reward-seeking behavior after presentation of the auditory cues) and does not generally affect reward-seeking behavior or the retrieval of the previously learned associations (i.e., reward-seeking behavior after presentation of the visual cues). Secondly, the neuronal activity measures will provide insights into the neural circuitry involved in memory inference (versus the retrieval of learned associations), and how this is influenced by stress hormone administration. Tests with auditory cues and visual cues cannot be combined within single animals for the following reasons: 1) the total number of non-reinforced trials (i.e., there is no reward outcome on the memory inference test) should to kept limited in order to prevent extinction of reward-seeking behavior; 2) for the analysis of neuronal activation after the memory inference test it is critical to discriminate between neuronal activation induced by memory inference (after presentation of the auditory cues) versus that induced by retrieval of the learned association (after presentation of the visual cues). The neural activity patterns will aid in determining the choice of target brain regions in DAP2 and DAP3.

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

Handling
Prior to the start of the behavioral protocols, the animals will be handled for 1-2 min on 3-4 consecutive days to habituate them to the experimenter and to reduce stress reactions to the different manipulations such as picking up and injection procedure. For the latter, the animals will be shortly restrained as necessary for the injection procedure but not given an actual injection.

Memory inference task (1)
During both the pre-training and the inference test protocols, mice will be allowed to explore an apparatus that is equipped with a speaker to present different auditory cues and a screen to present different visual cues. A liquid dispenser is used to deliver/remove the outcome which constitutes either a drop of 15% sucrose solution (reward; set 1) or a drop of water (neutral; set 2).

During the pre-training, mice will first complete an observational learning stage, conducted across 3-6 consecutive days. Each day, the mice will be placed in the apparatus for 15-20 sessions, each lasting□8-10 minutes. Each session will include 6 trials where an auditory cue is followed by presentation of the associated visual cue, from either set 1 or 2. The inter-trial interval (ITI) will be approximately 1.5 minutes. On each day of training, cues from set 1 and 2 will be presented equally often. To prepare for the next stage of the task (conditioning), across the final 2-3 days of the observational learning stage mice will be food restricted (see below). After the observational learning stage, the conditioning will be conducted across 4-5 consecutive days. Each day, the mice will be placed in the apparatus for 15-20 sessions, each lasting□8-10 minutes. Each session will include 6 trials where a visual cue is presented followed by the associated outcome, a drop of sucrose for set 1, or a drop of water for set 2. Each session will include cues from either set 1 or 2 ('blocked'), or from both set 1 and 2, presented in a pseudo-random order ('mixed'). Thus, in total mice will learn two auditory-visual cue associations in the observational learning stage and two visual cue-outcome associations in the conditioning stage. Pilot experiments will determine the optimal settings of the memory inference task.

After completion of the training protocol (observational learning and conditioning stages), mice will then proceed to the memory inference test (stage 3) where auditory cues (or visual cues to other groups of animals) will be presented in isolation for a total of 10 s, followed by an ITI of at least 30 s. Auditory cues (or visual cues) from set 1 and set 2 will be presented in a pseudo-random order, with a maximum of 30 trials. During the memory inference test, reward-seeking behavior will be quantified as the time spent in the outcome area in the 20 s period after the offset of the auditory cues (or visual cues). A reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2.

Food restriction
In the memory inference task, mice are trained to associate a visual cue (or auditory cue) with a rewarding outcome: a drop of sucrose (60 rewarded trials per day). Such a reward has only value when it fulfills a motivational need. In order to create such motivation, animals will be food restricted (to 90% of their free-feeding body weight) starting with a graduat transition of 2-3 days prior to the conditioning phase of the memory inference task. We will feed the animals daily after the learning task. The total duration of food restriction is 8-10 days. This food restriction regimen is the same as previously been used

successfully on the same memory inference task (1). This protocol is in accordance with the code of practice from the NCad on food restriction in neurocognitive research (2). We will regularly weigh the animals to keep track of their body weight. A drop of water is the non-rewarding control outcome, thus animals are not water restricted.

Stress hormone administration
The noradrenergic stimulant yohimbine (expected dose range: 0.3 - 3.0 mg/kg) or its saline vehicle, or CORT (expected dose range: 1 - 10 mg/kg) or its vehicle (5% ethanol in saline) will be administered by i.p. injection shortly (likely between 30 and 60 min) before the memory inference test. Drug dosages are based on extensive prior work in our and other laboratories examining yohimbine and CORT effects on other memory processes [redacted]. To control for possible effects of the injection procedure itself on behavior or neuronal activation (i.e., mice need to be restrained for a short period to give the i.p. injection), additional control groups are added that do not receive an injection.

Tissue collection
For the immunohistochemistry experiments, animals will be killed 30-90 min after the memory inference test by an overdose of anesthesia followed by transcardial perfusion with PBS and fixative.

General procedure
The essence of the memory inference test is that the animals have to join information acquired during the observational and conditioning stages that have not been observed together but lead to profitable outcomes. Therefore, repeated testing of the same animal is impossible. All comparisons will thus entail between-subject comparisons.

1. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183***, 228-243 (2020).
2. NCad Advies Motiveren door restricteren? https://ncadierproevenbeleid.nl/publicatie/18/6/5/nieuw-advies-ncad-motiveren-door-restriceren-uitgangspunten-voor-vocht-en-voedselinname.
[redacted]
4. Cai W.H. *et al.,* Postreactivation glucocorticoids impair recall of established fear memory. *J Neurosci* **26**, 9560-9566 (2006).

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

Based on published data on the memory inference task (1), we estimate to need 20 mice per group in the final analyses, but the exact number will be determined by power calculation using the expected effect sizes for each work protocol. Critical comparisons are made using mixed-model ANOVAs modelling the effect of drug treatment as between-subject variable, and reward-seeking bias for the auditory cues (or visual cues in control groups) (set 1 vs set 2) as within-subject variables. Reward-seeking behavior will be quantified as the time spent in the outcome area in the 20 s period after the offset of the auditory cues (or visual cues). The reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2 against chance.
Some animals may not acquire the task. As such, we will apply a drop-out rate of 10%, adding up to an initial 23 animals per group to reach the required n=20. We follow the general advice to round up 22.2 to 23 animals per group. Throughout all procedures, data drop-out and loss of animals will be minimized by careful execution of the experiments and close monitoring of animal welfare.

1. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183***, 228-243 (2020).

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
1	01	Charles River	8-20 weeks old	510	male	n/a	C57BL/6J

Provide justifications for these choices

Species

The mouse is a good model for investigating stress hormone effects of learning and memory because of its high translationability to humans. Further, previous studies have shown that mice are able to acquire the memory inference task.

Origin
Charles River Breeding facilities because it is a trusted animal breeder, and all our previous mouse studies were performed with animals from this breeder.

Life stages
Only adult animals (8-20 weeks) will be used, since we aim to assess the effects of stress hormone administration on mature behavior and brain function. Further, the brain atlas to determine coordinates for surgical procedures (in DAP2 and DAP3) is based on young adult animals.

Number
We request 23 animals per group. This includes 20 animals per group and as the average drop-out rate is expected to be 10%, the calculated number of animals needed is 22.2. We follow the general advice to round up 22.2 to 23 animals per group.

Pilot: As we have not previously worked with this memory inference task in our laboratory, prior to the real experiments we need to determine several of the settings such as the optimal number of training days for the observational (between 3 and 6 days) and conditioning phases (between 4 and 5 days), the number of sessions per day for each of these phases (between 15 and 20 sessions per day), the duration of each session (between 8 and 10 minutes), the nature of the visual and auditory cues of set 1 and set 2 such that the animals can discriminate between them. The pilot is considered successful if control animals exhibit a reward-seeking bias on the memory inference task. Further we need to determine the maximum number of non-reinforced trials (with a maximum of 30 trials) during the memory inference task without inducing extinction of reward-seeking behavior. Further, we will pilot the injection procedure as possible stressor such that if necessary we can adjust the handling sessions and injection method. If we observe a large difference in memory inference between non-injected and saline/vehicle-injected animals and we are unable to reduce this difference by adjusting the handling sessions or injection method, this will be considered a no-go criterium. These pilots will also allow new researchers to be trained on the procedures. We will only start with the actual experiments once the pilot has provided the optimal conditions. For these pilots, we will need a maximum of 50 animals. If we obtain the optimal settings with fewer animals, we will not use the remaining animals.

- **Yohimbine:** 5 drug conditions (3 drug doses + saline control + non-injection control) x 2 stimulus conditions (auditory cue or visual cue) = 10 groups
- **CORT:** 5 drug conditions (3 drug doses + vehicle control + non-injection control) x 2 stimulus conditions (auditory cue or visual cue) = 10 groups

Total number of animals: 510 mice (50 mice (pilot) + 20 groups x 23 mice).

Gender
Only male mice will be used for the following reasons: 1) to increase power by reducing inter-individual variability, since emotional arousal and stress responses in females have been shown to depend on their estrous cycle phase (1); and 2) because all data on which the working hypotheses are built were obtained in males.

1. Devall, A.J., Santos, J.M., Fry, J.P., Honour, J.W., Brandao, M.L., Lovick, T.A. Elevation of brain allopregnanolone rather than 5-HT release by short term, low dose fluoxetine treatment prevents the estrous cycle-linked increase in stress sensitivity in female rats. *Eur Neuropsychopharmacol* ***25,*** 113-123 (2015).

Genetic alterations
n/a

Strain
C57BL/6J mice will be used 1) because all data on which the working hypothesis are built were obtained in this strain of mice; 2) the memory inference task has been validated in this mouse strain 3) in future experiment we want to use transgenic animals with this background.

C. Accommodation and care
Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?
[] Yes

C. Accommodation and care

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

All animals will be single housed during the course of the experiment (starting from the first day of handling) for several reasons (approximate duration 3 weeks): Single housing will eliminate competition between animals when food is limited (during the last 8-10 days of the experiment). Further, the housing conditions (single vs grouped) are expected to affect behavior and the memory inference task has previously been validated only in single-housed animals. Moreover, all previous data acquired by our group on the effects of stress hormones on memory were obtained in single housed animals. Single housing will eliminate testing order effects and effects of social hierarchy that would be present in group-housed animals, and thereby reduce variance and increased power of the experimental design. Lastly, as in this DAP we will optimize several parameters (drug concentrations, training and test conditions et cetera) that will also be used in later DAPs (which require single housing because of surgery), it is important to keep housing conditions consistent across the different DAPs. Mice will be housed in conventional open lit cages in a colony room, thus the housing condition only prevents direct physical contact but no other social interactions via olfactory and auditory senses.
All animals will be food restricted (to 90% of their free-feeding body weight) starting with a gradual transition of 2-3 days prior to the conditioning phase of the memory inference task. Food restriction is needed in order to motivate the animals to perform the task and work for a reward. We will feed the animals daily after the learning task. The total duration of food restriction is 8-10 days. This protocol is in accordance with the code of practice from the NCad on food restriction in neurocognitive research (1). We will regularly weigh the animals to keep track of their body weight.

1. NCad Advies Motiveren door restricteren? https://ncadierproevenbeleid.nl/publicatie/18/6/5/nieuw-advies-ncad-motiveren-door-restriceren-uitgangspunten-voor-vocht-en-voedselinname.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

[X] No

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

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

The mice will experience moderate discomfort due to the single housing conditions for the length of the experiment (approx. 3 weeks). All mice will also receive a systemic drug administration (once) and an injection (for euthanasia) which both cause mild discomfort. Food restriction (for a maximum of 8-10 days) is also associated with mild discomfort and may cause a slight hunger feeling, but does not cause adverse effects.

Explain why these effects may emerge.

For the behavioral task the cause of the stress of the mice is primarily psychological (food restriction and reduced comfort by single housing). 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 and necessary for its success, we will take precautionary measures to minimize all other potential causes of (additional) stress to the animals. The animals will be handled prior to the experiments to get them used to human interventions. Perfusion will take place under deep anesthesia to minimize adverse effects.

E. 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 F.

E. Humane endpoints

[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 2 days, plus a criterion of 20% overall weight loss 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 humane 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 unlikely that any of the animals reach the humane endpoint over the course of the experiment. During the behavioral experiment, animals will be monitored routinely to check for standard humane endpoints and weighed 2-3 times per week to keep track of their body weight.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

The total (cumulative) discomfort of all animals on this DAP is expected to be moderate due to single housing (3 weeks), food restriction (a maximum of 8-10 days), behavioral task, systemic drug administration and pentobarbital injection.

G. 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 complex interaction between stress (hormones), brain function and behavior cannot be investigated with animal-free alternatives. Animal studies are indispensable to provide causal evidence for the neural mechanisms underlying stress hormone effects on memory flexibility, which cannot be done in humans. The mouse is the lowest animal species in which we are able to attain behavioral models that are comparable to humans. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and exact stimulus exposure.

Reduction
The requested number of animals (20 animals per group for the final analyses) is based on group sizes reported in literature for the same readout and is the minimum required for reliable statistical conclusions. The behavioral and neural readout parameters will be performed in the same animals, which saves animals and simultaneously increases the power of the measurements. In our experimental design, we included non-injection control groups to determine whether the stress associated with the injection procedure by itself has an effect on behavior. If we will find that the injection procedure does not affect behavior on this task, then we will no longer include this group in further experiments. As described in the general design, all other experimental groups are absolutely necessary to answer the experimental question.

Refinement
Pilot studies will determine the optimal conditions for the actual experiments. The experiments will be carried out with the least discomfort possible. The memory inference task is associated with minimal discomfort and can be an enrichment for the animals (boredom is a source of suffering). Animals will be mildly food restricted to motivate them for the cognitive task. Mild food restriction (up to about 90% of free feeding body weight) is better for health than *ad libitum* feeding. Furthermore, the experiments require single housing of the animals (3 weeks), which may cause moderate discomfort due to isolation, but will prevent that the dominant animal eats the food of the subordinate animals, eliminate test-order effects and reduce stress-induced fighting that may occur between cage mates. Cages will be enriched with extra bedding material and a house to provide comfort.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.
[X] No
[] Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

H. Re-use
Will animals be used that have already been used in other animal procedures ?
[X] No > Continue with question I.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

I. RepetitionExplain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.
n/a

J. 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 K.
[] 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.

End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

[] No > Provide information on the destination of the animals.

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

The brain tissue is necessary for further analysis.

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

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

[X] Yes > Will a method of killing be used for which specific requirements apply?

[X] No > Describe the method of killing.

Mice will be killed by pentobarbital injection followed by transcardial perfusion.

[] Yes > Describe the method of killing that will be used and provide justifications for this choice.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

n/a

Centrale Commissie Dierproeven

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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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 serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number 2	Type of animal procedure Determining the effect of local administration of stress hormones into specific brain areas on memory flexibility

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
In this part of the project, we will examine aim 2 of the project proposal: to determine the effects of norepinephrine (NE) and corticosterone (CORT), and their functional interaction, in specific brain regions on memory flexibility and the neuronal representation of memory flexibility.

We will have two experiments:

In **Experiment 1**, we will examine the effect of either NE or CORT administration into specific brain regions on memory inference. All mice will undergo stereotaxic surgery for implantation of bilateral guide cannulas which will allow for accurate local administration into the brain. We will target a maximum of three different brain regions: Based on our hypothesis (Project Proposal, Figure 1) and current knowledge from mainly human neuroimaging studies, the dorsal hippocampus, ventral hippocampus, prefrontal cortex (PFC) and amygdala are prime candidates, but a final selection will be made after we obtain the

neuronal activity findings of DAP1. After the postsurgical recovery period (>1 week), they will be trained on the memory inference task (see project proposal, section 3.4.1, for a schematic representation of the general design of the task). The exact experimental settings will be informed by DAP1. Shortly before the memory inference test (stage 3), NE or a glucocorticoid will be administered via the implanted cannula. A maximum of three different dosages of NE or glucocorticoid (or their respective vehicles) will be tested. As explained in DAP1, three different dosages are needed to examine the complex dose-response effects (moderate doses have an effect on memory whereas lower or higher doses are ineffective). The procedure of the memory inference test will be identical as described in DAP1: We will present auditory cues from set 1 and set 2 in isolation, without visual cues or outcomes, and measure evidence for inference from the auditory cue to outcome by quantifying reward-seeking behavior, defined as the time spent in the outcome area in the 20 s period after the offset of the auditory cues. A reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2. The reward-seeking bias is the primary outcome parameter for the behavioral measure of memory inference. Also similar as explained in DAP1, other groups of mice (given the same drug treatments) will be presented visual cues from set 1 and 2 in isolation, without auditory cues or outcomes. These control groups are necessary to verify that the stress hormone administration selectively affects memory inference and does not induce general effects on the animals' incentive to seek the reward or affect the retrieval of the learned association (visual cue-outcome). All mice will be killed by perfusion fixation 30-90 min after the memory inference test for later immunohistochemistry experiments and to check for correct cannula placement. Neuronal readouts will be acquired by measuring different immediate early gene (IEG) expression responses after the memory inference test and stress hormone administration.

In **Experiment 2**, we will examine functional interactions between NE and CORT on memory inference. Evidence from several studies indicates that such functional interactions between NE and CORT may be essential in regulating different memory functions. For example, an attenuation of NE signaling with a β-adrenoceptor antagonist administered into several different brain regions blocks CORT effects on both the consolidation and retrieval of memory. By contrast, an attenuation of CORT signaling with a glucocorticoid receptor antagonist resulted in that a much higher dose of NE was needed to modulate memory. These findings indicate that CORT modulates these memory functions by having a permissive influence on the noradrenergic system. However, we recently found that the effects of NE and CORT are not always synergistic and that they can have opposite effects on other memory functions. We do presently not know whether NE and CORT interact in influencing memory flexibility. Also in this experiment, all mice will first undergo surgery for cannula implantation, and after recovery (>1 week) they will be trained on the memory inference task. Shortly before the memory inference test (stage 3), mice will be administered three different dosages of NE (or saline control) together with a glucocorticoid receptor antagonist (or vehicle control) via the implanted cannula. Other groups of animals will receive three different dosages of a glucocorticoid together with a noradrenergic antagonist. A maximum of three different dosages of the agonist are needed here to determine whether the antagonist fully blocks the agonist effect or whether it induces a shifts in the dose-response effects such that either a higher or lower dose of the agonist now becomes effective. Depending on the findings of these experiments, additional experiments might be required (e.g., if the glucocorticoid receptor antagonist abolishes the effect of NE administration, it might be required to determine whether similar effects are observed by blocking the mineralocorticoid receptor). We likely will examine these functional interactions only within a single brain region. All mice will be killed by perfusion fixation 30-90 min after the memory inference test for later immunohistochemistry experiments and to check for correct cannula placement. Figure 1 shows the timeline and general design of the experiments on this DAP.

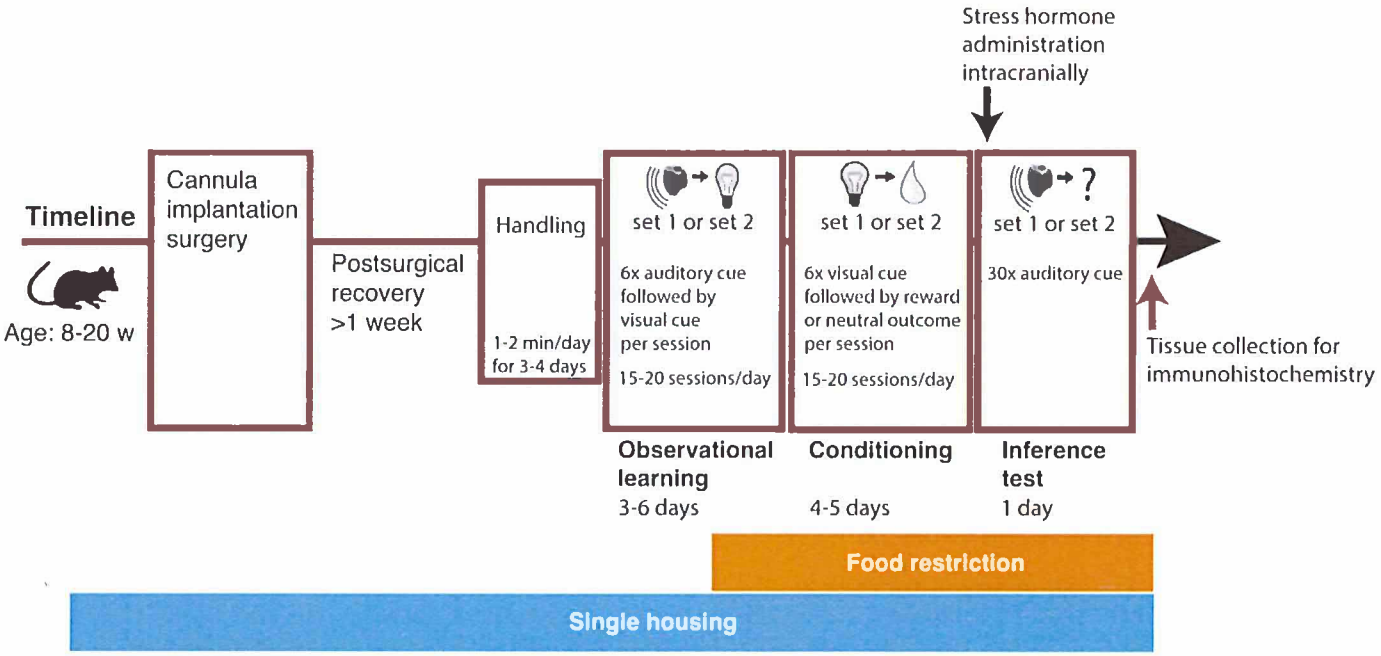


Figure 1: *Timeline and general design of the experiments on this DAP.*

Primary outcome parameters
- Behavioral measure of memory inference: The reward-seeking bias in response to the auditory cues quantified as the difference in reward-seeking behavior for set 1 and set 2.
- Neuronal activity measure: Immunohistochemistry is used to measure the IEG expression in brain after the memory inference test and stress hormone administration.

Justification
Experiment 1 will provide insight into the local actions of the two stress hormones NE and CORT on the behavioral measure of memory inference. The neuronal activity measures will provide insights into how local stress hormone administration into these brain regions influences neuronal activity in other brain regions and thus affect functional connectivity during the memory inference test. The neuronal activity patterns will also determine the choice for targeting specific brain circuits in DAP3. Experiment 2 will provide insight into whether the effects of NE and CORT on memory inference depend on functional interactions between these two stress hormone systems. These findings will provide important insight into the working mechanisms of NE and CORT on memory flexibility and whether these are likely to exert common effects on the underlying neural circuitry (which is important for determining the exact design of the experiments in DAP3). As explained in DAP1, the presentation of auditory cues and visual cues (in different groups of animals) during the memory inference test is required to verify that the stress hormone administration specifically impacts memory inference (i.e., reward-seeking behavior after presentation of the auditory cues) and does not generally affect reward-seeking behavior or the retrieval of the previously learned associations (i.e., reward-seeking behavior after presentation of the visual cues). Tests with auditory cues and visual cues cannot be combined within single animals for the following reasons: 1) the total number of non-reinforced trials (i.e., there is no reward outcome on the memory inference test) should to kept limited in order to prevent extinction of reward-seeking behavior; 2) for the analysis of neuronal activation after the memory inference test it is critical to discriminate between neuronal activation induced by memory inference (after presentation of the auditory cues) versus that induced by retrieval of the learned association (after presentation of the visual cues).

Of note, it is unlikely that we will perform all experimental conditions described in this DAP, as their necessity depends on the results of previous DAPs. Nevertheless, due to the high potential value of the results, we find it essential to describe and include all possible conditions here, and evaluate their necessity after performing the experiments of previous DAPs (selection point).

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

Surgery
All mice will be anesthetized with isoflurane and positioned in the stereotaxic apparatus. An analgesic will be given via their drinking water from 24 h prior to surgery until at least 48 h post-surgery in order to minimize discomfort. Further, a local analgesic will be administered on the periost/scalp before making an incision. Guide cannulas will be implanted bilaterally and affixed to the skull. Structured stereotaxic

techniques and a highly standardized protocol for both the surgical procedure as well as pre-, peri-, and post-operative care steps will be followed (1). Starting 2-3 days prior to surgery, mice will be individually housed throughout the remainder of the experiment. Animals will be allowed to recover for >1 week.

Handling
Prior to the start of the behavioral protocols, the animals will be handled for 1-2 min on 3-4 consecutive days to habituate them to the experimenter and to reduce stress reactions to the different manipulations such as picking up and intracranial injection procedure. For the latter, the animals will be shortly restrained as necessary for the injection procedure but not given an actual injection.

Memory inference task (2)
During both the pre-training and the inference test protocols, mice will be allowed to explore an apparatus that is equipped with a speaker to present different auditory cues and a screen to present different visual cues. A liquid dispenser is used to deliver/remove the outcome which constitutes either a drop of 15% sucrose solution (reward; set 1) or a drop of water (neutral; set 2).

During the pre-training, mice will first complete an observational learning stage, conducted across 3-6 consecutive days. Each day, the mice will be placed in the apparatus for 15-20 sessions, each lasting8-10 minutes. Each session will include 6 trials where an auditory cue is followed by presentation of the associated visual cue, from either set 1 or 2. The inter-trial interval (ITI) will be approximately 1.5 minutes. On each day of training, cues from set 1 and 2 will be presented equally often. To prepare for the next stage of the task (conditioning), across the final 2-3 days of the observational learning stage mice will be food restricted (see below). After the observational learning stage, the conditioning will be conducted across 4-5 consecutive days. Each day, the mice will be placed in the apparatus for 15-20 sessions, each lasting8-10 minutes. Each session will include 6 trials where a visual cue is presented followed by the associated outcome, a drop of sucrose for set 1, or a drop of water for set 2. Each session will include cues from either set 1 or 2 ('blocked'), or from both set 1 and 2, presented in a pseudo-random order ('mixed'). Thus, in total mice will learn two auditory-visual cue associations in the observational learning stage and two visual cue-outcome associations in the conditioning stage. The optimal settings of the memory inference task will be determined in DAP1.

After completion of the training protocol (observational learning and conditioning stages), mice will then proceed to the memory inference test (stage 3) where auditory cues (or visual cues to other groups of animals) will be presented in isolation for a total of 10 s, followed by an ITI of at least 30 s. Auditory cues (or visual cues) from set 1 and set 2 will be presented in a pseudo-random order, with a maximum of 30 trials. During the memory inference test, reward-seeking behavior will be quantified as the time spent in the outcome area in the 20 s period after the offset of the auditory cues (or visual cues). A reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2.

Food restriction
In the memory inference task, mice are trained to associate a visual cue (or auditory cue) with a rewarding outcome: a drop of sucrose (60 rewarded trials per day). Such a reward has only value when it fulfills a motivational need. In order to create such motivation, animals will be food restricted (to 90% of their free-feeding body weight) starting with a graduat transition of 2-3 days prior to the conditioning phase of the memory inference task. We will feed the animals daily after the learning task. The total duration of food restriction is 8-10 days. This food restriction regimen is the same as previously been used successfully on the same memory inference task (1). This protocol is in accordance with the code of practice from the NCad on food restriction in neurocognitive research (2). We will regularly weigh the animals to keep track of their body weight. A drop of water is the non-rewarding control outcome, thus animals are not water restricted.

Local Drug Administration
Stress hormone (or antagonist) infusions or an equivalent volume of control solution will be made using 30-gauge injection needles connected to 10-µl Hamilton microsyringes by polyethylene (PE-20) tubing. Drug will be administered shortly (likely between 30 and 60 min) before the memory inference test. Mice will be gently restrained to insert the infusion needles into the guide cannula. Solutions will then be injected slowly by a minipump and the injection needles will be retained within the cannulas for 20 s after drug infusion to maximize diffusion.

Tissue collection
For the validation of cannula placement and immunohistochemistry experiments, animals will be killed 30-90 min after the memory inference test by an overdose of anesthesia followed by transcardial perfusion with PBS and fixative.

General procedure
The essence of the memory inference test is that the animals have to join information acquired during the observational and conditioning stages that have not been observed together but lead to profitable

outcomes. Therefore, repeated testing of the same animal is impossible. All comparisons will thus entail between-subject comparisons.

1. Fornari, R.V. et al. Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience. *J Vis Exp* **59** (2012).
2. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183***, 228-243 (2020).
3. NCad Advies Motiveren door restricteren? https://www.ncadierproevenbeleid.nl/documenten/publicatie/18/6/5/nieuw-advies-ncad-motiveren-door-restriceren-uitgangspunten-voor-vocht-en-voedselinname.

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

Based on published data on the memory inference task (1), we estimate to need 20 mice per group in the final analyses, but the exact number will be determined by power calculation using the expected effect sizes for each work protocol. Critical comparisons are made using mixed-model ANOVAs modelling the effect of drug treatment as between-subject variable, and reward-seeking bias for the auditory cues (or visual cues in control groups) (set 1 vs set 2) as within-subject variables. Reward-seeking behavior will be quantified as the time spent in the outcome area in the 20 s period after the offset of the auditory cues (or visual cues). The reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2 against chance.
Cannula implantation into a slightly incorrect brain region will lead to exclusion of the animal (as this causes suboptimal manipulation of the target region). In addition, some animals may not acquire the task. As such, we will apply a drop-out rate of 20%, adding up to an initial 25 animals per group to reach the required n=20. Throughout all procedures, data drop-out and loss of animals will be minimized by careful execution of the experiments and close monitoring of animal welfare.

1. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183***, 228-243 (2020).

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
2	01	Charles River	8-20 weeks old	2040	male	n/a	C57BL/6J

Provide justifications for these choices

Species
The mouse is a good model for investigating stress hormone effects of learning and memory because of its high translationability to humans. Further, previous studies have shown that mice are able to acquire the memory inference task.

Origin
Charles River Breeding facilities because it is a trusted animal breeder, and all our previous mouse studies were performed with animals from this breeder.

Life stages
Only adult animals (8-20 weeks) will be used, since we aim to assess the effects of stress hormone administration on mature behavior and brain function. Further, the brain atlas to determine coordinates for surgical procedures is based on young adult animals.

Number
We request 25 animals per group. This includes 20 animals per group and as the average drop-out rate is expected to be 20%, we request 5 additional animals per group.

Pilot: We need to determine the optimal coordinates for local injections in the brain sites, determine the optimal drug dosage, and practice the surgical procedure to minimize drop-out (due to incorrect injection position). We will only start with the actual experiments once the pilot has provided the optimal conditions. For this pilot experiment, we will need a maximum of 40 animals (n=10 for each of the 3 brain regions + n=10 to determine the optimal doses of the glucocorticoid receptor and noradrenergic antagonist).

Experiment 1:
- 8 drug conditions (3 NE doses + saline control and 3 glucocorticoid doses + vehicle control) x 2 stimulus conditions (auditory cue or visual cue) x 3 brain regions = 48 groups

Experiment 2:
- NE + glucocorticoid receptor antagonist: 8 drug conditions (3 NE doses + saline control x 1 glucocorticoid antagonist + vehicle control) x 2 stimulus conditions (auditory cue or visual cue) = 16 groups
- Glucocorticoid + noradrenergic antagonist: 8 drug conditions (3 glucocorticoid doses + vehicle control x 1 noradrenergic antagonist + saline control) x 2 stimulus conditions (auditory cue or visual cue) = 16 groups

Total number of animals: 2040 mice (40 mice (pilot) + 80 groups x 25 mice).

Gender
Only male mice will be used for the following reasons: 1) to increase power by reducing inter-individual variability, since emotional arousal and stress responses in females have been shown to depend on their estrous cycle phase (1); and 2) because all data on which the working hypotheses are built were obtained in males.

1. Devall, A.J., Santos, J.M., Fry, J.P., Honour, J.W., Brandao, M.L., Lovick, T.A. Elevation of brain allopregnanolone rather than 5-HT release by short term, low dose fluoxetine treatment prevents the estrous cycle-linked increase in stress sensitivity in female rats. *Eur Neuropsychopharmacol* ***25,*** 113-123 (2015).

Genetic alterations
n/a

Strain
C57BL/6J mice will be used 1) because all data on which the working hypothesis are built were obtained in this strain of mice; 2) the memory inference task has been validated in this mouse strain 3) in future experiment we want to use transgenic animals with this background.

C. Accommodation and care
Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?
[] Yes
[X] No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

All animals will be single housed during the course of the experiment (starting from 2-3 days prior to the surgery) for several reasons (approximate duration 4-5 weeks): Following the surgery, the cannula will be closed off with a metal pin. Other animals in the cage would be able to remove this pin, which on the one hand would expose the cannula and on the other hand could potentially harm (the pin is sharp) the animals. Single housing will also eliminate competition between animals when food is limited (during the last 8-10 days of the experiment). Further, the housing conditions (single vs grouped) are expected to affect behavior and the memory inference task has previously been validated only in single-housed animals. Moreover, all previous data acquired by our group on the effects of stress hormones on memory were obtained in single housed animals. Lastly, single housing will eliminate testing order effects and effects of social hierarchy that would be present in group-housed animals, and thereby reduce variance and increased power of the experimental design. Mice will be housed in conventional open lit cages in a colony room, thus the housing condition only prevents direct physical contact but no other social interactions via olfactory and auditory senses.
All animals will be food restricted (to 90% of their free-feeding body weight) starting with a gradual transition of 2-3 days prior to the conditioning phase of the memory inference task. Food restriction is needed in order to motivate the animals to perform the task and work for a reward. We will feed the animals daily after the learning task. The total duration of food restriction is 8-10 days. This protocol is in accordance with the code of practice from the NCad on food restriction in neurocognitive research (1). We will regularly weigh the animals to keep track of their body weight.

1. NCad Advies Motiveren door restricteren? https://ncadierproevenbeleid.nl/publicatie/18/6/5/nieuw-advies-ncad-motiveren-door-restriceren-uitgangspunten-voor-vocht-en-voedselinname.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

[] No

[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.

The animal facility has standard operating procedures for mouse surgery.
Anesthesia: isoflurane (4%) during induction, after which isoflurane levels are reduced and maintained at 1.5-2%.
Analgesia: rimadyl in drinking water from 24 h before surgery until at least 48 after surgery.
Lidocaine solution, combined with bupivacaine, will be administered locally before making an incision, and also on periost/scalp before cleaning scalp & drilling.

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

The surgery will not cause any discomfort since the operation will be performed under anesthesia. The mice will experience moderate discomfort during the recovery period (approx. 3-5 days), and single housing conditions for the length of the experiment (approx. 4-5 weeks). All rats will further be given intracranial drug administration and an injection (for euthanasia) which causes mild discomfort. Food restriction (for a maximum of 8-10 days) is also associated with mild discomfort and may cause a slight hunger feeling, but does not cause adverse effects.

Explain why these effects may emerge.

For the behavioral task the cause of the stress of the mice is primarily psychological (food restriction and reduced comfort by single housing). These stressors are however necessary for these experiments to succeed. For the surgery, primarily psychological as pain is suppressed by analgesics. However, the surgery is necessary for the experiments.

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

Although some stressors are inherent to the experimental design and necessary for its success, we will take precautionary measures to minimize all other potential causes of (additional) stress to the animals. The animals will be handled prior to the experiments to get them used to human interventions. During the surgery (performed by trained researchers) we will closely monitor vital signs and take the necessary measures (optimizing the dose of anesthesia) to minimize discomfort. In addition, analgesic will be provided for optimal pain relief and recovery. Perfusion will take place under deep anesthesia to minimize adverse effects.

E. 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 F.

[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 2 days, plus a criterion of 20% overall weight loss 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. Further, the situation where the guide cannulas come off is also considered a humane endpoint.

*Standard humane 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.

Although unlikely (based on previous experience <2%), the first 2-3 days of the recovery phase after surgery is the most critical period at which humane endpoints are reached. During this period, animals may show reduced food intake and reduced locomotor or grooming behavior. Therefore, animals will be monitored daily until 3 days post-surgery for signs of sickness, infection, excessive weight loss (>15% in 2 days, plus a criterion of 20% overall weight loss), or other signs of diminished well-being. Also during the behavioral experiment, animals will be monitored routinely to check for standard humane endpoints and weighed 2-3 times per week to keep track of their body weight. Overall expected incidence of humane endpoint prior to the finalization of the experiment is low.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

The total (cumulative) discomfort of all animals on this DAP is expected to be moderate due to the surgery and single housing (4-5 weeks). The food restriction (a maximum of 8-10 days), behavioral task, drug administration and pentobarbital injection are expected to cause mild discomfort.

G. 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 complex interaction between stress (hormones), brain function and behavior cannot be investigated with animal-free alternatives. Animal studies are indispensable to provide causal evidence for the neural mechanisms underlying stress hormone effects on memory flexibility, which cannot be done in humans. The mouse is the lowest animal species in which we are able to attain behavioral models that are comparable to humans. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and exact stimulus exposure. Furthermore, stress hormone administration directly into specific brain regions is not possible in humans.

Reduction

The requested number of animals (20 animals per group for the final analyses) is based on group sizes reported in literature for the same readout and is the minimum required for reliable statistical conclusions. The behavioral and neural readout parameters will be performed in the same animals, which saves animals and simultaneously increases the power of the measurements. Of note, it is unlikely that we will perform all experimental conditions described in this DAP, as their necessity depends on the results of previous DAPs. Nevertheless, due to the high potential value of the results, we find it essential to describe and include all possible conditions here, and evaluate their necessity after performing the experiments of previous DAPs (selection point).

Refinement

Pilot studies will determine the optimal conditions for the actual experiments. The experiments will be carried out with the least discomfort possible. Standardized pre-, peri- and post-operative care guidelines will be followed. The memory inference task is associated with minimal discomfort and can be an enrichment for the animals (boredom is a source of suffering). Animals will be mildly food restricted to motivate them for the cognitive task. Mild food restriction (up to about 90% of free feeding body weight) is better for health than *ad libitum* feeding. Furthermore, the experiments require single housing of the animals (4-5 weeks), which may cause moderate discomfort due to isolation, but will prevent potential physical damage that could occur by removal of the sharp pin that is enclosed in the cannula by a cage mate and that the dominant animal eats the food of the subordinate animals. Further, single housing will eliminate test-order effects and reduce stress-induced fighting that may occur between cage mates. Cages will be enriched with extra bedding material and a house to provide comfort.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

[X] No

[] Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

[X] No > Continue with question I.

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

I. RepetitionExplain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

n/a

J. 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 K.

J. Location where the animals procedures are performed

[] 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.

End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

[] No > Provide information on the destination of the animals.

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

The brain tissue is necessary for further analysis.

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

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

[X] Yes > Will a method of killing be used for which specific requirements apply?

[X] No > Describe the method of killing.

Mice will be killed by pentobarbital injection followed by transcardial perfusion.

[] Yes > Describe the method of killing that will be used and provide justifications for this choice.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

n/a

Centrale Commissie Dierproeven

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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on 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 serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number 3	Type of animal procedure Providing causal evidence for the modulation of neural systems in regulating stress hormone effects on memory flexibility

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
In this part of the project, we will examine aim 3 of the project proposal: to provide causal evidence for the modulation of neural circuit function in the establishment of the effects of norepinephrine (NE) and corticosterone (CORT) on memory flexibility.

We will have three experiments:

In **Experiment 1**, we will test the hypothesis that NE and CORT impair memory flexibility by suppressing activity of the prefrontal cortex (PFC) and hippocampus (see project proposal, section 3.1, for a schematic representation of this hypothesis). For this, we will combine DREADD (designer receptor exclusively activated by designer drugs) technology to selectively activate or silence different sites within the PFC and hippocampus with systemic stress hormone manipulations. By bidirectionally manipulating neural activity in these sites we can determine both whether an activation of either the PFC or hippocampus is necessary to block the impairing effects of NE and CORT on memory flexibility and whether silencing of these brain

sites is sufficient to mimic (and thus occlude) the impairing effects of NE and CORT on memory flexibility. Demonstrating both necessity and sufficiency is a requirement to prove causality. Different groups of mice will be injected bilaterally with an adeno-associated virus encoding the respective DREADD receptors (e.g., hM4D, hM3D, or control) into the PFC, dorsal hippocampus or ventral hippocampus. After the postsurgical recovery period (>2 week), they will be trained on the memory inference task (see project proposal, section 3.4.1, for a schematic representation of the general design of the task) (2). The exact experimental settings will be informed by DAP1. Shortly before the memory inference test (stage 3), a nimals will receive systemic administration of a low dose of the cognate ligand clozapine to activate the DREADD receptors. Similar to DAP1, animals will also receive peripheral administration of yohimbine, CORT or their respective vehicles (saline for yohimbine, 5% ethanol in saline for corticosterone). Only a single, optimal dose of each hormone will be administered here; the dosage shown to be most effective in impairing memory flexibility as determined in DAP1. The procedure of the memory inference test will be identical as described in DAP1 and DAP2: We will present auditory cues from set 1 and set 2 in isolation, without visual cues or outcomes, and measure evidence for inference from the auditory cue to outcome by quantifying reward-seeking behavior, defined as the time spent in the outcome area in the 20 s period after the offset of the auditory cues. A reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2. The reward-seeking bias is the primary outcome parameter for the behavioral measure of memory inference. As explained in DAP1 and DAP2, other groups of mice (with the same experimental manipulations) will be presented visual cues from set 1 and 2 in isolation, without auditory cues or outcomes. These control groups are necessary to verify that the experimental manipulations selectively affect memory inference and do not induce general effects on the animals' incentive to seek the reward or affect the retrieval of the learned association (visual cue-outcome). To analyze changes in neural activity in other brain region, the mice will be killed by perfusion fixation 30-90 min after the memory inference test for later immunohistochemistry experiments and to ensure specific viral targeting placement. Neuronal readouts will be acquired by measuring different immediate early gene (IEG) expression responses to the stress hormone and neural circuit manipulations and testing. Thereby, this series of experiments will provide causal and mechanistic evidence for the critical recruitment of the PFC and hippocampus in mediating the effects of NE and CORT on impairing memory flexibility.

In **Experiment 2**, we will test the hypothesis that NE and CORT impair memory flexibility by reducing functional connectivity between the PFC and hippocampus (see project proposal, section 3.1, for a schematic representation of this hypothesis). Similar as described for Experiment 1, by both activating and silencing PFC-hippocampal functional connectivity we can determine whether an activation of the PFC-hippocampal circuit blocks the impairing effects of NE and CORT on memory flexibility whereas a silencing of this circuit mimics the impairing effects of NE and CORT can thereby causes an occlusion of the stress hormone effects. However, as there are no direct monosynaptic projections from the PFC to the hippocampus, we will use two different approaches to manipulate PFC-hippocampal functional connectivity. In **Experiment 2a**, we will employ a well-established symmetrical/asymmetrical manipulation approach of PFC and hippocampus activity (Figure 1). Mice will be injected unilaterally with an adeno-associated virus encoding the respective DREADD receptors (e.g., hM4D, hM3D, or control) into the PFC (left and right will be counterbalanced) and a second injection into either the contralateral or ipsilateral hippocampus (dorsal or ventral hippocampus, depending on the findings of the first experiment) The asymmetrical manipulation (i.e., contralateral injection into the PFC and hippocampus) will affect PFC-hippocampal connectivity in both hemispheres, whereas the symmetrical manipulation (i.e., ipsilateral injection into the PFC and hippocampus) will remain PFC-hippocampal connectivity in one hemisphere intact and thus serves as control. In **Experiment 2b,** we will inject the adeno-associated virus encoding the respective DREADD receptors (e.g., hM4D, hM3D, or control) bilaterally into the nucleus reuniens (NRE), which is an important anatomical link between the PFC and hippocampus. After recovery, the animals will be trained on the memory inference task. Shortly before the memory inference test (stage 3), animals will receive systemic administration of the cognate ligand clozapine as well as a single dose of yohimbine, CORT or their respective vehicles (saline for yohimbine, 5% ethanol in saline for CORT) (same as in Experiment 1). If the findings of the first experiment will indicate that the manipulation of the PFC and hippocampus selectively affects memory inference and not retrieval of the learned visual cue-outcome association, then in this second experiment we only will test animals with the auditory cue (and not the visual cue). To analyze changes in neural activity, the mice will be killed by perfusion fixation 30-90 min after the inference test for later immunohistochemistry experiments and to ensure specific viral targeting placement. Thereby, this series of experiments will provide causal and mechanistic evidence for the critical recruitment for PFC-hippocampal circuits in mediating the effects of NE and CORT on memory flexibility.

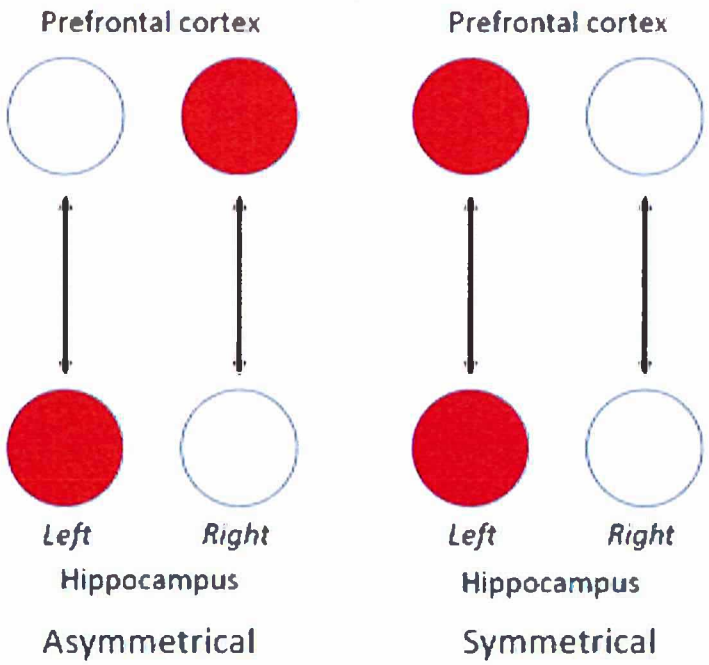


Figure 1: *Schematic of the design of Experiment 2a with asymmetrical and symmetrical manipulations of the prefrontal cortex (PFC) and hippocampus. With asymmetrical unilateral manipulation of the PFC and hippocampus, functional connectivity between the PFC and hippocampus is bilaterally affected, whereas with symmetrical unilateral manipulation of the PFC and hippocampus, functional connectivity is affected only in one of the two hemispheres. Red = manipulated regions.*

In **Experiment 3**, we will test the hypothesis that amygdala projections to both the PFC and hippocampus are critically involved in mediating the effects of NE and CORT on memory flexibility (see project proposal, section 3.1, for a schematic representation of this hypothesis). Mice will be injected bilaterally with a retrograde adeno-associated virus expressing Cre recombinase into the target area (i.e., PFC or hippocampus), while an inhibitory or excitatory Cre-dependent adeno-associated virus coding the respective DREADD receptors (e.g., hM4D, hM3D, or control) will be injected bilaterally into the amygdala. The exact targets (e.g., dorsal or ventral hippocampus) will be determined based on the findings of DAP1-3. The combined administration of these two viruses will allow for the specific targeting of those projection neurons in which both viruses are concurrently expressed. After recovery, the animals will be trained on the memory inference task. Shortly before the memory inference test (stage 3), animals will receive systemic administration of the cognate ligand clozapine as well as a single dose of yohimbine, CORT or their respective vehicles (saline for yohimbine, 5% ethanol in saline for CORT) (same as in Experiments 1 and 2). If the findings of the first experiment will indicate that the manipulation of the PFC and hippocampus selectively affects memory inference and not retrieval of the learned visual cue-outcome association, then in this third experiment we will only test animals with the auditory cue (and not the visual cue). To analyze changes in neural activity, the mice will be killed by perfusion fixation 30-90 min after the inference test for later immunohistochemistry experiments and to ensure specific viral targeting placement. Figure 2 shows the timeline and general design of the experiments on this DAP.

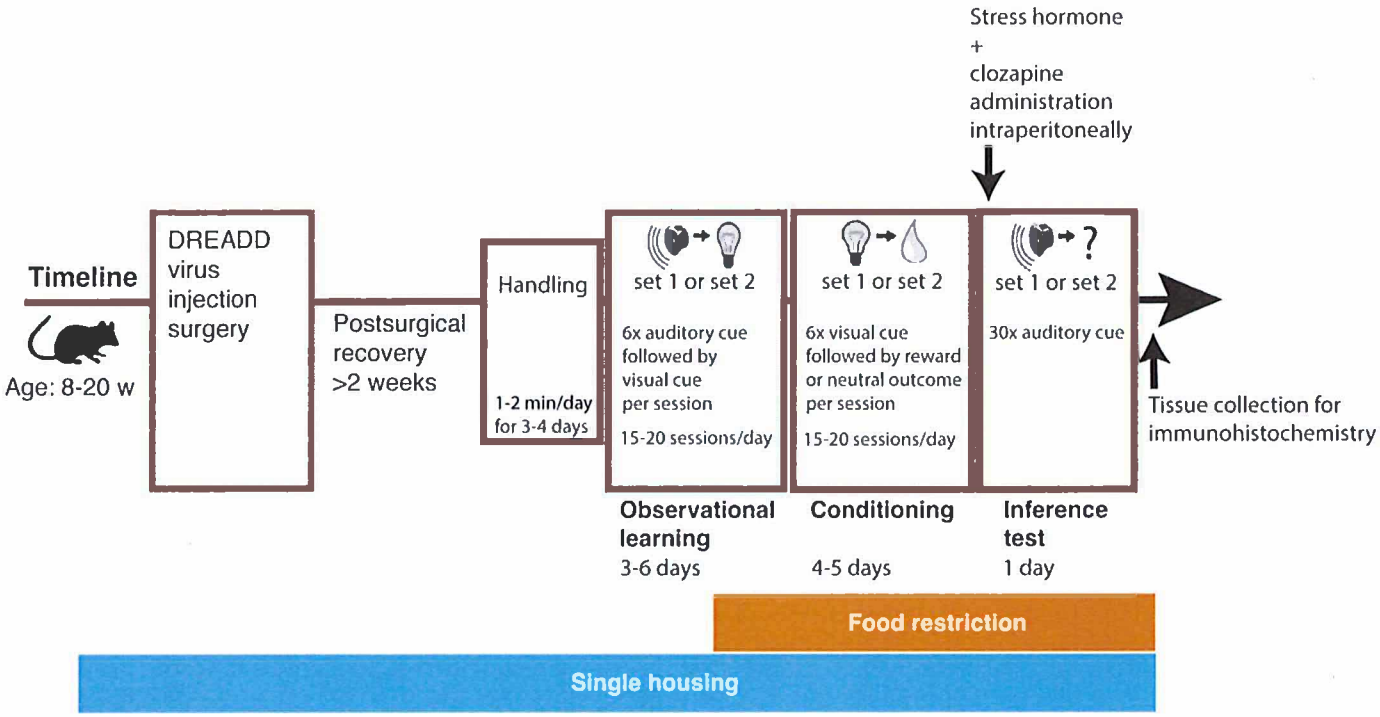


Figure 2: *Timeline and general design of the experiments on this DAP.*

Primary outcome parameters
- Behavioral measure of memory inference: The reward-seeking bias in response to the auditory cues quantified as the difference in reward-seeking behavior for set 1 and set 2.
- Neuronal activity measure: Immunohistochemistry is used to measure the IEG expression in brain after the memory inference test and stress hormone administration.

Justification
The findings in these experiments will provide causal and mechanistic evidence for the critical recruitment of specific brain regions and pathways in mediating the effects of the stress hormones NE and CORT on memory inference. As explained in DAP1, the presentation of auditory cues and visual cues (in different groups of animals) during the memory inference test is required to verify that the neural circuit and stress hormone manipulations specifically impact memory inference (i.e., reward-seeking behavior after presentation of the auditory cues) and do not generally affect reward-seeking behavior or the retrieval of the previously learned associations (i.e., reward-seeking behavior after presentation of the visual cues). Tests with auditory cues and visual cues cannot be combined within single animals. The neuronal activity measures are needed to determine the specificity and success rate of the viral manipulations in altering neural activity within the target brain regions or neural circuits.

Of note, it is unlikely that we will perform all experimental conditions described in this DAP, as their necessity depends on the results of previous DAPs. Nevertheless, due to the high potential value of the results, we find it essential to describe and include all possible conditions here, and evaluate their necessity after performing the experiments of previous DAPs (selection point).

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

Surgery
All mice will be anesthetized with isoflurane and positioned in the stereotaxic apparatus. An analgesic will be given via their drinking water from 24 h prior to surgery until at least 48 h post-surgery in order to minimize discomfort. Further, a local analgesic will be administered on the periost/scalp before making an incision. In order to manipulate neural activity within the PFC, hippocampus and nucleus reuniens (Experiment 1 and 2), mice will be injected with an excitatory or inhibitory DREADD virus which' expression is specific for either glutamatergic (e.g., pAAV-CaMKIIa-hM3D/hM4D□mCherry or control virus pAAV-CaMKIIa-mCherry) or GABAergic (e.g., pAAV-hDlx-GqDREADD/GiDREADD-dTomato-Fishell-4/5 or control virus pAAV-hDlx-Flex-dTomato-Fishell_7) neurons. To manipulate amygdala projections to the PFC and hippocampus (Experiment 3), an retrograde AAV virus will be injected bilaterally into either the PFC or hippocampus and an anterograde DREADD virus (e.g., AAV9-hSyn-DIO-hM4D/hM3D-mCherry or the control virus AAV9-DIOmCherry) will be bilaterally injected into the amygdala. Structured stereotaxic techniques and a highly standardized protocol for both the surgical procedure as well as pre-, peri-, and

post-operative care steps will be followed (1). Starting 2-3 days prior to surgery, mice will be individually housed throughout the remainder of the experiment. Animals will be allowed to recover for 1-2 weeks to ensure sufficient viral expression (2).

Handling
Prior to the start of the behavioral protocols, the animals will be handled for 1-2 min on 3-4 consecutive days to habituate them to the experimenter and to reduce stress reactions to the different manipulations such as picking up and injection procedure. For the latter, the animals will be shortly restrained as necessary for the injection procedure but not given an actual injection.

Memory inference task (3)
During both the pre-training and the inference test protocols, mice will be allowed to explore an apparatus that is equipped with a speaker to present different auditory cues and a screen to present different visual cues. A liquid dispenser is used to deliver/remove the outcome which constitutes either a drop of 15% sucrose solution (reward; set 1) or a drop of water (neutral; set 2).

During the pre-training, mice will first complete an observational learning stage, conducted across 3-6 consecutive days. Each day, the mice will be placed in the apparatus for 15-20 sessions, each lasting 8-10 minutes. Each session will include 6 trials where an auditory cue is followed by presentation of the associated visual cue, from either set 1 or 2. The inter-trial interval (ITI) will be approximately 1.5 minutes. On each day of training, cues from set 1 and 2 will be presented equally often. To prepare for the next stage of the task (conditioning), across the final 2-3 days of the observational learning stage mice will be food restricted (see below). After the observational learning stage, the conditioning will be conducted across 4-5 consecutive days. Each day, the mice will be placed in the apparatus for 15-20 sessions, each lasting 8-10 minutes. Each session will include 6 trials where a visual cue is presented followed by the associated outcome, a drop of sucrose for set 1, or a drop of water for set 2. Each session will include cues from either set 1 or 2 ('blocked'), or from both set 1 and 2, presented in a pseudo-random order ('mixed'). Thus, in total mice will learn two auditory-visual cue associations in the observational learning stage and two visual cue-outcome associations in the conditioning stage. The optimal settings of the memory inference task will be determined in DAP1.

After completion of the training protocol (observational learning and conditioning stages), mice will then proceed to the memory inference test (stage 3) where auditory cues (or visual cues to other groups of animals) will be presented in isolation for a total of 10 s, followed by an ITI of at least 30 s. Auditory cues (or visual cues) from set 1 and set 2 will be presented in a pseudo-random order, with a maximum of 30 trials. During the memory inference test, reward-seeking behavior will be quantified as the time spent in the outcome area in the 20 s period after the offset of the auditory cues (or visual cues). A reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2.

Food restriction
In the memory inference task, mice are trained to associate a visual cue (or auditory cue) with a rewarding outcome: a drop of sucrose (60 rewarded trials per day). Such a reward has only value when it fulfills a motivational need. In order to create such motivation, animals will be food restricted (to 90% of their free-feeding body weight) starting with a graduat transition of 2-3 days prior to the conditioning phase of the memory inference task. We will feed the animals daily after the learning task. The total duration of food restriction is 8-10 days. This food restriction regimen is the same as previously been used successfully on the same memory inference task (1). This protocol is in accordance with the code of practice from the NCad on food restriction in neurocognitive research (2). We will regularly weigh the animals to keep track of their body weight. A drop of water is the non-rewarding control outcome, thus animals are not water restricted.

Clozapine administration
An effective low dose of clozapine will be dissolved in 1 M hydrochloric acid and 0.1 M PBS, and then administered by i.p. injection shortly (likely between 30 and 60 min) before the memory inference test (5).

Stress hormone administration
The noradrenergic stimulant yohimbine (0.3 - 3.0 mg/kg) or its saline vehicle, or CORT (1 - 10 mg/kg) or its vehicle (5% ethanol in saline) will be administered by i.p. injection shortly (likely between 30 and 60 min) before the memory inference test. Only a single dosage shown to be most effective in influencing memory inference (as determined in DAP1) will be used.

Tissue collection
For the validation of the viral expression as well as for the immunohistochemistry experiments, animals will be killed 30-90 min after the memory inference test by an overdose of anesthesia followed by transcardial perfusion with PBS and fixative.

General procedure
The essence of the memory inference test is that the animals have to join information acquired during the observational and conditioning stages that have not been observed together but lead to profitable outcomes. Therefore, repeated testing of the same animal is impossible. All comparisons will thus entail between-subject comparisons.

1. Fornari, R.V. et al., Rodent stereotaxic surgery and animal welfare outcome improvements for behavioral neuroscience. *J Vis Exp* **59** (2012).
2. Aschauer D.F. et al., Analysis of transduction efficiency, tropism and axonal transport of AAV serotypes 1, 2, 5, 6, 8 and 9 in the mouse brain. *PloS One* ***8*** (2013).
3. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183***, 228-243 (2020).
4. NCad Advies Motiveren door restricteren? https://www.ncadierproevenbeleid.nl/documenten/publicatie/18/6/5/nieuw-advies-ncad-motiveren-door-restricteren-uitgangspunten-voor-vocht-en-voedselinname.
5. Zerbi, V. et al., Rapid reconfiguration of the functional connectome after chemogenetic locus coeruleus activation. *Neuron* ***103,*** 702-718 (2019).

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

Based on published data on the memory inference task (1), we estimate to need 20 mice per group in the final analyses, but the exact number will be determined by power calculation using the expected effect sizes for each work protocol. Critical comparisons are made using mixed-model ANOVAs modelling the effect of drug treatment and DREADD manipulation as between-subject variables, and reward-seeking bias for the auditory cues (or visual cues in control groups) (set 1 vs set 2) as within-subject variables. Reward-seeking behavior will be quantified as the time spent in the outcome area in the 20 s period after the offset of the auditory cues (or visual cues). The reward-seeking bias will be quantified as the difference in reward-seeking behavior for set 1 and set 2 against chance.
Virus injection into a slightly incorrect brain region will lead to exclusion of the animal (as this causes suboptimal manipulation of the target region). In addition, some animals may not acquire the task. As such, we will apply a drop-out rate of 20%, adding up to an initial 25 animals per group to reach the required n=20. Throughout all procedures, data drop-out and loss of animals will be minimized by careful execution of the experiments and close monitoring of animal welfare.

1. Barron, H.C. et al., Neuronal computation underlying inferential reasoning in humans and mice. *Cell* ***183***, 228-243 (2020).

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
3	01	Charles River	8-20 weeks old	3350	male	n/a	C57BL/6J

Provide justifications for these choices

Species
The mouse is a good model for investigating stress hormone effects of learning and memory because of its high potential of translation to humans. Further, previous studies have shown that mice are able to acquire the memory inference task.

Origin
Charles River Breeding facilities because it is a trusted animal breeder, and all our previous mouse studies were performed with animals from this breeder.

Life stages
Only adult animals (8-20 weeks) will be used, since we aim to assess the effects of stress hormone administration on mature behavior and brain function. Further, the brain atlas to determine coordinates for surgical procedures is based on young adult animals.

Number

We request 25 animals per group. This includes 20 animals per group and as the average drop-out rate is expected to be 20%, we request 5 additional animals per group.

Pilot: We need to determine the optimal coordinates and infusion volume for viral injections in the brain sites (PFC, dorsal hippocampus, ventral hippocampus, BLA and nucleus reuniens), viral titer, incubation time as well as dose of clozapine. Previous findings indicate that the optimal setting for these parameters can vary depending on the specific DREADD virus used. We will use previously established protocols to validate the optimal settings: We will test the optimal coordinates, infusion volume, viral titer and incubation time by checking histologically whether the virus injection induces sufficient transfection (visualized as fluorescent signal) within the target region without spreading to neighboring regions. We will check the effectiveness of clozapine by administering different doses of clozapine prior to the memory inference test and then determine the dose that induces a maximal suppression or activation (depending on the type of DREADD virus and compared to a control virus) in IEG expression within transfected cells by immunohistochemistry. We will only start with the actual experiments once the pilot has provided the optimal conditions. For this pilot experiment, we will need a maximum of 50 animals (n=10 per brain region). If we obtain the optimal settings with fewer animals, we will not use the remaining animals.

Experiment 1:
- 4 drug conditions (1 NE dose + saline control and 1 CORT dose + vehicle control) x 3 viruses (activation/inhibition/control) x 3 brain regions x 2 stimulus conditions (auditory cue or visual cue) = 72 groups

Experiment 2:
- Experiment 2a: 4 drug conditions (1 NE dose + saline control and 1 CORT dose + vehicle control) x 3 viruses (activation/inhibition/control) x 2 conditions (symmetrical or asymmetrical) = 24 groups
- Experiment 2b: 4 drug conditions (1 NE dose + saline control and 1 CORT dose + vehicle control) x 3 viruses (activation/inhibition/control = 12 groups

Experiment 3:
- Amygdala-PFC pathway: 4 drug conditions (1 NE dose + saline control and 1 CORT dose + vehicle control) x 3 viruses (activation/inhibition/control) = 12 groups
- Amygdala-hippocampus pathway: 4 drug conditions (1 NE dose + saline control and 1 CORT dose + vehicle control) x 3 viruses (activation/inhibition/control) = 12 groups

Total number of animals: 3350 mice (50 mice (pilot) + 132 groups x 25 mice).

Gender
Only male mice will be used for the following reasons: 1) to increase power by reducing inter-individual variability, since emotional arousal and stress responses in females have been shown to depend on their estrous cycle phase (1); and 2) because all data on which the working hypotheses are built were obtained in males.

1. Devall, A.J., Santos, J.M., Fry, J.P., Honour, J.W., Brandao, M.L., Lovick, T.A. Elevation of brain allopregnanolone rather than 5-HT release by short term, low dose fluoxetine treatment prevents the estrous cycle-linked increase in stress sensitivity in female rats. *Eur Neuropsychopharmacol* ***25,*** 113-123 (2015).

Genetic alterations
n/a

Strain
C57BL/6J mice will be used 1) because all data on which the working hypothesis are built were obtained in this strain of mice; 2) the memory inference task has been validated in this mouse strain 3) in future experiment we want to use transgenic animals with this background.

C. Accommodation and care
Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?
[] Yes
[X] No > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

All animals will be single housed during the course of the experiment (starting from 2-3 days prior to surgery) for several reasons (approximate duration 4-5 weeks): Single housing will eliminate competition between animals when food is limited (during the last 8-10 days of the experiment). Further, the housing conditions (single vs grouped) are expected to affect behavior and the memory inference task has previously been validated only in single-housed animals. Moreover, all previous data acquired by our group on the effects of stress hormones on memory were obtained in single housed animals. Single

housing will eliminate testing order effects and effects of social hierarchy that would be present in group-housed animals, and thereby reduce variance and increased power of the experimental design. Lastly, it is important to keep housing conditions consistent across the different DAPs to maximize comparison of results between the different parts of this project. Mice will be housed in conventional open lit cages in a colony room, thus the housing condition only prevents direct physical contact but no other social interactions via olfactory and auditory senses.
All animals will be food restricted (to 90% of their free-feeding body weight) starting with a gradual transition of 2-3 days prior to the conditioning phase of the memory inference task. Food restriction is needed in order to motivate the animals to perform the task and work for a reward. We will feed the animals daily after the learning task. The total duration of food restriction is 8-10 days. This protocol is in accordance with the code of practice from the NCad on food restriction in neurocognitive research (1). We will regularly weigh the animals to keep track of their body weight.

1. NCad Advies Motiveren door restricteren? https://ncadierproevenbeleid.nl/publicatie/18/6/5/nieuw-advies-ncad-motiveren-door-restriceren-uitgangspunten-voor-vocht-en-voedselinname.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

[] No

[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.

The animal facility has standard operating procedures for mouse surgery.
Anesthesia: isoflurane (4%) during induction, after which isoflurane levels are reduced and maintained at 1.5-2%.
Analgesia: rimadyl in drinking water from 24 h before surgery until at least 48 after surgery.
Lidocaine solution, combined with bupivacaine, will be administered locally before making an incision, and also on periost/scalp before cleaning scalp & drilling.

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

The surgery will not cause any discomfort since the operation will be performed under anesthesia. The mice will experience moderate discomfort during the recovery period (approx. 3-5 days), and single housing conditions for the length of the experiment (approx. 4-5 weeks). All rats will further be given systemic drug administration and an injection (for euthanasia) which causes mild discomfort. Food restriction (for a maximum of 8-10 days) is also associated with mild discomfort and may cause a slight hunger feeling, but does not cause adverse effects.

Explain why these effects may emerge.

For the behavioral task the cause of the stress of the mice is primarily psychological (food restriction and reduced comfort by single housing). These stressors are however necessary for these experiments to succeed. For the surgery, pain is suppressed by analgesics. However, the surgery is necessary for the experiments.

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

Although some stressors are inherent to the experimental design and necessary for its success, we will take precautionary measures to minimize all other potential causes of (additional) stress to the animals. The animals will be handled prior to the experiments to get them used to human interventions. During the surgery (performed by trained researchers) we will closely monitor vital signs and take the necessary measures (optimizing the dose of anesthesia) to minimize discomfort. In addition, analgesic will be provided for optimal pain relief and recovery. Perfusion will take place under deep anesthesia to minimize adverse effects.

E. Humane endpoints

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

E. Humane endpoints

[] No > Continue with question F.

[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 2 days, plus a criterion of 20% overall weight loss 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 humane 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.

Although unlikely (based on previous experience <2%), the first 2-3 days of the recovery phase after surgery is the most critical period at which humane endpoints are reached. During this period, animals may show reduced food intake and reduced locomotor or grooming behavior. Therefore, animals will be monitored daily until 3 days post-surgery for signs of sickness, infection, excessive weight loss (>15% in 2 days, plus a criterion of 20% overall weight loss), or other signs of diminished well-being. Also during the behavioral experiment, animals will be monitored routinely to check for standard humane endpoints and weighed 2-3 times per week to keep track of their body weight. Overall expected incidence of humane endpoint prior to the finalization of the experiment is low.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

The total (cumulative) discomfort of all animals on this DAP is expected to be moderate due to the surgery and single housing (4-5 weeks). The food restriction (a maximum of 8-10 days), behavioral task, drug administration and pentobarbital injection are expected to cause mild discomfort.

G. 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 complex interaction between stress (hormones), brain function and behavior cannot be investigated with animal-free alternatives. Animal studies are indispensable to provide causal evidence for the neural mechanisms underlying stress hormone effects on memory flexibility, which cannot be done in humans. The mouse is the lowest animal species in which we are able to attain behavioral models that are comparable to humans. In comparison with humans, the mouse offers the possibility to precisely control environmental conditions, such as the timing and exact stimulus exposure.

Reduction
The requested number of animals (20 animals per group for the final analyses) is based on group sizes reported in literature for the same readout and is the minimum required for reliable statistical conclusions. The behavioral and neural readout parameters will be performed in the same animals, which saves animals and simultaneously increases the power of the measurements. Of note, it is unlikely that we will perform all experimental conditions described in this DAP, as their necessity depends on the results of previous DAPs. Nevertheless, due to the high potential value of the results, we find it essential to describe and include all possible conditions here, and evaluate their necessity after performing the experiments of previous DAPs (selection point).

Refinement
Pilot studies will determine the optimal conditions for the actual experiments. The experiments will be carried out with the least discomfort possible. Standardized pre-, peri- and post-operative care guidelines will be followed. The memory inference task is associated with minimal discomfort and can be an enrichment for the animals (boredom is a source of suffering). Animals will be mildly food restricted to motivate them for the cognitive task. Mild food restriction (up to 90% of free feeding body weight) is better for health than *ad libitum* feeding. Furthermore, the experiments require single housing of the animals (4-5 weeks), which may cause moderate discomfort due to isolation, but will prevent that the dominant animal eats the food of the subordinate animals, eliminate test-order effects and reduce stress-induced fighting that may occur between cage mates. Cages will be enriched with extra bedding material and a house to provide comfort.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.
[X] No
[] Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

H. Re-use
Will animals be used that have already been used in other animal procedures ?
[X] No > Continue with question I.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

I. RepetitionExplain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.
n/a

J. 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 K.
[] 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.

End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

[] No > Provide information on the destination of the animals.

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

The brain tissue is necessary for further analysis.

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

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

[X] Yes > Will a method of killing be used for which specific requirements apply?

[X] No > Describe the method of killing.

Mice will be killed by pentobarbital injection followed by transcardial perfusion.

[] Yes > Describe the method of killing that will be used and provide justifications for this choice.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

n/a

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD10300 2022 16030 / 2022-0008
2. Titel van het project: Neural mechanisms underlying stress hormone effects on memory flexibility
3. Titel van de NTS: De effecten van stresshormonen op geheugenflexibiliteit

4. Type aanvraag:
   ☒ nieuwe aanvraag projectvergunning
   ☐ wijziging van vergunning met nummer

5. Contactgegevens DEC:
   - naam DEC: RUDEC
   - telefoonnummer contactpersoon: [redacted] bereikbaar op maandag, dinsdag, en donderdag van 9:00 tot 15:00 uur
   - e-mailadres contactpersoon: dierexperimentencommissie@radboudumc.nl

6. Adviestraject (data dd-mm-jjjj):
   ☒ ontvangen door DEC: 08-07-2022, eerder ontvangen op 09-05-2022
   ☐ aanvraag compleet
   ☒ in vergadering besproken: 12-07-2022
   ☐ anderszins behandeld
   ☒ termijnonderbreking(en): Geen onderbreking
   ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   ☐ aanpassing aanvraag
   ☒ advies aan CCD: 25-07-2022

7. De inhoud van dit project is afgestemd met de IvD en deze heeft geen bezwaren tegen de uitvoering van het project binnen deze instelling.

8. Eventueel horen van aanvrager:
   - Datum: 14-06-2022
   - Plaats: Nijmegen
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager: [redacted]
   - Gestelde vraag / vragen: De aanvrager is verzocht een toelichting te geven op het onderzoek beschreven in de vergunningaanvraag en aan te geven hoe dit past in zijn onderzoekslijn. De onderzoeker legt uit dat effecten van stresshormonen op accuraatheid en gedetailleerdheid van het geheugen al eerder bij knaagdieren zijn onderzocht. Flexibiliteit van geheugen, soms noodzakelijk voor een adequate reactie op een nieuwe situatie, is echter nog nooit bij dieren onderzocht, waardoor er nog niets bekend is over de achterliggende mechanismen en eventuele betrokkenheid van hersengebieden. De aanvrager heeft in zijn toelichting tevens aandacht besteed aan een deel van het schriftelijke commentaar dat hij via de CCD heeft ontvangen. De commissie heeft geen aanvullende schriftelijke vragen gesteld naar aanleiding van de presentatie. Wel is de onderzoeker verzocht om alle opmerkingen en vragen van de commissie, ontvangen via de CCD, te adresseren in een herziene versie van de projectaanvraag.
   - Het horen van de aanvrager heeft geleid tot het indienen van een herziene aanvraag.

9. Correspondentie met de aanvrager:

10. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er is geen betrokkenheid van DEC-leden bij deze projectaanvraag, waardoor onafhankelijkheid en onpartijdigheid zijn gewaarborgd.

C. Beoordeling (inhoud)

1. Deze basaal wetenschappelijke aanvraag richt zich op het bestuderen van het effect van stresshormonen op flexibiliteit van het geheugen van muizen. Het is bekend dat stresshormonen (adrenaline en noradrenaline; corticosteron) de opslag van het geheugen voor de betreffende gebeurtenis/situatie versterken. Tegelijk bemoeilijken noradrenaline en corticosteron het ophalen van herinneringen aan eerdere gebeurtenissen/situaties. Voor het afleiden van onbekende verbanden, noodzakelijk voor het nemen van beslissingen in nieuwe situaties, worden herinneringen aan eerdere ervaringen flexibel samengevoegd. Deze geheugen flexibiliteit is essentieel om gedrag aan een nieuwe situatie aan te kunnen passen. Recent is duidelijk geworden dat stress niet alleen de accuraatheid en gedetailleerdheid van geheugen beïnvloedt, maar ook de flexibiliteit van geheugen vermindert. Een gebrek aan geheugenflexibiliteit is een kenmerk van PTSS: de herinnering aan het trauma wordt gekenmerkt door weinig contextuele details en starheid, waardoor blootstelling aan trauma-gerelateerde 'cues' (geur, geluid) resulteren in sterke emotionele reacties en herbelevingen. Deze sterke emotionele reacties bemoeilijken mogelijk de behandeling van PTSS, en mogelijk ook van andere stress-gerelateerde aandoeningen. De mechanismen achter geheugenflexibiliteit, en de (interactie tussen de) betrokken hersencircuits zijn echter nog niet opgehelderd. In deze aanvraag worden deze mechanismen bestudeerd en de betrokkenheid/rol van prefrontale cortex – hippocampus circuits en de amygdala (en de manier waarop ze elkaar beïnvloeden) onderzocht. Het onderzoek is verdeeld in drie logische onderdelen die elkaar grotendeels opvolgen, waarbij eerst wordt bepaald of systemische manipulatie van stresshormonen een meetbaar effect heeft op de uitkomsten van de gekozen gedragstaak om flexibiliteit van geheugen te meten. De commissie gaat er vanuit dat yohimbine alleen in lagere doseringen gebruikt zal worden, waardoor alleen effecten die verlopen via de alfa2-adrenerge receptoren worden geblokkeerd. Het is dan niet nodig om selectievere antagonisten (bijvoorbeeld atipamezole) te gebruiken. Tevens wordt in het eerste deel van het onderzoek duidelijk, door 'immediate early gene' analyse direct na afloop van de geheugentaak, welke hersengebieden daarbij betrokken zijn. Vervolgens worden stresshormonen lokaal aan geselecteerde hersengebieden toegediend, en wordt het effect daarvan op flexibiliteit van geheugen bepaald. Tenslotte wordt getracht causaliteit vast te stellen van de gevonden relaties met behulp van zogenaamde designer drugs die bepaalde hersencircuits specifiek kunnen stimuleren of dempen. Indien er geen waarneembaar effect is (in uitkomsten van de gekozen gedragstaak) van de stresshormonen op geheugen flexibiliteit (de eerste stap), dan zullen de vervolgstappen niet worden uitgevoerd. De commissie constateert op grond daarvan dat deze aanvraag een concrete, goed afgebakende doelstelling heeft en getypeerd kan worden als een project. De opzet komt het best overeen met voorbeeld 1 uit de 'Handreiking invulling definitie project'. De verschillende subdoelen volgen elkaar logisch op, en leveren informatie die gebruikt wordt voor

het ontwerp van experimenten voor het volgende subdoel. Tezamen geven alle subdoelen een beeld van de onderzochte mechanismen en betrokken hersengebieden. Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan. De aanvrager heeft, zowel binnen de doelstellingen en bijlagen dierproeven, als tussen de doelstellingen, beschreven op basis van welke criteria hij zal besluiten het project voort te zetten. De DEC is er daardoor van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en er niet onnodig dieren gebruikt zullen worden. Gezien bovenstaande is de DEC van mening dat de aanvraag toetsbaar is en voldoende samenhang heeft.

2. Voor zover de DEC weet is er geen "tegenstrijdige" wetgeving die het uitvoeren van de experimenten in de weg zou kunnen staan.

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

   Belangen en waarden

4. Het directe doel van het project is te achterhalen hoe de stresshormonen NA en CORT de flexibiliteit van het geheugen van muizen beïnvloeden, en welke hersencircuits daarbij betrokken zijn. Het uiteindelijke doel is beter begrip van de invloed van stress op geheugenflexibiliteit, zodat die kennis gebruikt kan worden voor de ontwikkeling van behandelingen voor mensen die lijden aan stress-gerelateerde mentale aandoeningen, zoals PTSS en angststoornissen. De mechanismen die een rol spelen bij de reactie op stress zijn evolutionair gezien goed geconserveerd. Het is daarom aannemelijk dat mechanismen die bij muizen een rol spelen, ook van belang zijn voor effecten van stress op het geheugen van mensen, en de manier waarop die stressreactie flexibiliteit van geheugen beïnvloedt. Parallel aan het beschreven onderzoek met muizen vindt onderzoek met proefpersonen plaats, hetgeen translatie uit beide onderzoeksgebieden faciliteert. Er is daarom binnen deze aanvraag een vrij directe relatie tussen het doel van deze projectaanvraag en het uiteindelijke doel. De aanvrager heeft duidelijk gemaakt dat de kennis van de mechanismen die ten grondslag liggen aan geheugenflexibiliteit en het effect van stresshormonen daarop bij muizen nog zeer beperkt is, dat deze kennis nodig is voor de ontwikkeling van nieuwe behandelingen voor stress-gerelateerde psychische stoornissen, en dat er behoefte is aan nieuwe behandelingen. Naar de mening van de DEC is het doel van deze projectaanvraag daarom gerechtvaardigd binnen de context van het onderzoeksveld.

5. De belangrijkste belanghebbenden in deze projectaanvraag zijn de proefdieren, de onderzoekers, de wetenschappelijke gemeenschap, en de doelgroep/patiënten.
   Voor de proefdieren geldt dat hun welzijn en integriteit worden aangetast (zie C11 en C12). De dieren zullen beperkt worden in hun natuurlijke gedrag en gedurende de proeven zullen de dieren stress ondervinden en pijn ondergaan. Uiteindelijk zullen ze in het kader van het onderzoek gedood worden. De dieren hebben er belang bij hiervan gevrijwaard te blijven.
   Voor de onderzoekers geldt dat het publiceren van belangrijke nieuwe wetenschappelijke inzichten resulteert in een goede wetenschappelijke reputatie, hetgeen vaak de sleutel is voor het verkrijgen van nieuwe onderzoeksmiddelen en mogelijkheden. Dit kan door de onderzoeker en de betrokken organisaties zelf van belang geacht worden, maar dient naar de mening van de DEC geen rol te spelen in de ethische afweging over de toelaatbaarheid van het gebruik van proefdieren. Het gaat uiteindelijk om de vraag of dit onderzoek belangrijke maatschappelijke en wetenschappelijke doelen dient (gezondheid, kennis).
   Voor de wetenschappelijke gemeenschap (met name neurowetenschappers en cognitieve

psychologen) is dit onderzoek van belang omdat het nieuwe kennis zal opleveren over de mechanismen achter flexibiliteit van geheugen, over de betrokken hersengebieden, en de invloed van stresshormonen daarop. Geheugenflexibiliteit speelt bij veel gedrag een rol. Kennis over effecten van stress daarop, kunnen daarom van belang zijn voor keuzes omtrent de opzet van onderzoek waarin geheugenflexibiliteit van belang is. De gedragstaak en het theoretische model kunnen door andere onderzoekers gebruikt worden in hun onderzoek.
Voor patiënten met PTSS is dit onderzoek indirect en op de lange termijn van belang, omdat het kan leiden tot de ontwikkeling van betere diagnostiek en/of nieuwe behandelingen voor hun aandoeningen (met name niet-adaptieve herinneringen). De huidige behandelingen zijn voor meer dan de helft van PTSS-patiënten niet effectief genoeg. Gerichte behandeling op basis van mechanistisch inzicht in de effecten van stresshormonen op hersenen en flexibiliteit van geheugen zou kunnen bijdragen aan de ontwikkeling van effectievere behandelingen voor mensen die lijden aan (niet-adaptieve) emotionele herinneringen. Dit kan er toe leiden dat de patiënt weer gezond wordt, dan wel een betere kwaliteit van leven heeft. De resultaten zijn waarschijnlijk ook relevant voor de ontwikkeling van effectievere therapieën voor andere stress-gerelateerde psychische stoornissen, zoals angststoornissen. Kunnen beschikken over adequate behandelingen voor stress-gerelateerde psychische stoornissen is van groot belang voor de samenleving.

6. Er is geen aanleiding voor de DEC om de bewering van de aanvrager dat er geen nadelige effecten voor het milieu te verwachten zijn, in twijfel te trekken.

Proefopzet en haalbaarheid

7. De kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven zijn voldoende gewaarborgd. De aanvrager heeft zeer veel ervaring met onderzoek naar effecten van stresshormonen op geheugenfunctie van knaagdieren. Het onderzoek heeft geresulteerd in tal van publicaties in goede wetenschappelijke tijdschriften. De commissie is daarom overtuigd van de kwaliteit van het werk van de aanvrager. De aanvrager beschikt over voldoende kennis en kunde, onder andere op grond van een artikel 9 kwalificatie, om te kunnen voldoen aan alle zorgvuldigheidseisen omtrent het verrichten van dierproeven.

8. De voorgestelde experimentele opzet en uitkomstparameters sluiten logisch aan bij de doelstellingen van het project (zie C1 en C4). Bovendien heeft deze groep veel ervaring in dit onderzoeksveld en met de voorgestelde dierproeven. De doelstellingen van het project in zijn huidige vorm zijn ambitieus: het betreft grote aantallen dieren die bijvoorbeeld hersenoperaties ondergaan en vervolgens uitgebreid geanalyseerd worden. De commissie mist een goede onderbouwing van de logistieke haalbaarheid van het project in deze vorm. De onderzoeker heeft tijdens zijn mondelinge presentatie aangegeven dat er gedurende het onderzoek keuzes gemaakt zullen worden, waardoor het aannemelijk is dat er minder dieren gebruikt zullen worden, maar dit is niet expliciet opgenomen in de projectaanvraag.
De aanvrager is zich ervan bewust dat de voor dit onderzoek benodigde intraperitoneale injecties (inclusief het fixeren van de dieren voor die injecties) ook stressvol zijn voor de dieren. Door de dieren uitgebreid te hanteren waarbij ze kunnen wennen aan de fixatie wordt deze stress zoveel mogelijk tegengaan. Zoals ook met de onderzoeker tijdens de mondelinge presentatie in juni uitvoerig besproken, vermoedt de commissie dat er in voorgaande jaren soortgelijke studies door zijn onderzoeksgroep gedaan zijn waaruit blijkt hoe lang het effect daarvan aanhoudt (bijvoorbeeld door het meten van bloedwaardes van corticosteron). De commissie had het erg gewaardeerd wanneer de onderzoeker zijn mening hierover had onderbouwd met data, met name omdat ook niet duidelijk is aangegeven in de DAPs hoe kort de injecties voor de flexibele

geheugen tests worden toegediend. In de projectaanvraag is weliswaar een adequaat no-go criterium ingebouwd dat het onderzoek stopt als blijkt dat door die handelingen opgewekte stress inderdaad teveel interfereert met de hoofdexperimenten, maar reeds bestaande kennis daarover zou van belang kunnen zijn voor vroegtijdige optimalisatie van de experimentele omstandigheden. De DEC is van mening dat het project goed is opgezet, en dat deze strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project.

Welzijn dieren

9. Er is geen sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   ☐ Bedreigde diersoort(en) (10e lid 4)
   ☐ Niet-menselijke primaten (10e)
   ☐ Dieren in/uit het wild (10f)
   ☐ Niet gefokt voor dierproeven (11, bijlage I richtlijn)
   ☐ Zwerfdieren (10h)
   ☐ Hergebruik (1e lid 2)
   ☐ Locatie: buiten instelling vergunninghouder (10g)
   ☐ Geen toepassing verdoving/pijnbestrijding (13)
   ☐ Dodingsmethode niet volgens bijlage IV richtlijn (13c lid 3)

10. In de aanvraag wordt, om redenen van dierenwelzijn of diergezondheid of om redenen die samenhangen met de proefopzet afgeweken van de eisen in bijlage III van richtlijn 2010/63/EU betreffende de huisvesting en verzorging van de dieren. De aanvrager geeft daarvoor de volgende reden(en): om de dieren te motiveren voor de gedragstaak, worden zij gedurende acht tot tien dagen beperkt gevoerd (voedselrestrictie), waarbij zij dagelijks gevoerd worden na training van de gedragstaak. Deze methode is eerder gebruikt bij deze gedragstaak (uitgevoerd door andere onderzoekers in een ander laboratorium). De onderzoeker is op de hoogte van de 'Code of practice' van de NCad, waarin de randvoorwaarden voor voedselrestrictie in neurocognitief onderzoek worden vermeld. Alle dieren worden gedurende maximaal vijf weken individueel gehuisvest vanaf 2-3 dagen voor de operatieve implantatie van canules of vanaf de eerste dag dat de dieren gehanteerd worden door de onderzoeker. De canules worden afgesloten met een pin, die door een kooimaat verwijderd zou kunnen worden waardoor de canule niet langer beschermd is en de dieren zich zouden kunnen bezeren aan de scherpe pin. Individuele huisvesting is bovendien noodzakelijk wanneer de dieren beperkt gevoerd worden (voedselrestrictie) om te voorkomen dat het dominante dier in de kooi het meeste voer eet. Bovendien zijn alle eerder verzamelde data over de effecten van stresshormonen op geheugen verkregen bij individueel gehuisveste dieren. Tenslotte, individuele huisvesting elimineert test-volgorde effecten en effecten van sociale hiërarchie, waardoor variantie wordt verminderd en de power van het experimentele design toeneemt. De DEC is van mening dat de gegeven redenen voldoende onderbouwing hiervoor zijn. De dieren worden wel individueel gehuisvest, maar zijn niet sociaal geïsoleerd van soortgenoten (zij kunnen die wel zien, horen en ruiken).

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd als matig voor alle 5900 muizen. Het ongerief wordt hoofdzakelijk veroorzaakt door individuele huisvesting gedurende enkele weken, een stereotactische operatie voor het aanbrengen van twee canules, een i.p. injectie, voedselrestrictie, en (training voor) een gedragstaak om flexibiliteit van geheugen te meten. De commissie is van mening dat de inschatting van het cumulatieve ongerief logisch volgt uit de beschreven handelingen met de dieren.

12. De integriteit van dieren wordt aangetast door het instrumentele gebruik van de dieren dat inherent is aan het doen van dierproeven. Bovendien worden bij de meeste dieren canules in de hersenen gebracht om lokaal stresshormonen of antagonisten daarvan toe te kunnen dienen. Het dier wordt hierdoor gehinderd in zijn normale gedrag en de zelfredzaamheid neemt af.

13. De criteria voor humane eindpunten zijn voldoende specifiek gedefinieerd en toegesneden op het experiment. Het percentage dieren dat naar verwachting een humaan eindpunt zal bereiken is op basis van eigen ervaring en gegevens uit de wetenschappelijke literatuur ingeschat. De commissie is het eens met deze inschatting en de gehanteerde humane eindpunten.

3V's

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn. De complexe interactie tussen stresshormonen, hersenfunctie en gedrag kan niet met proefdiervrije alternatieven onderzocht worden. De in deze aanvraag beschreven manipulaties en analyses kunnen niet bij mensen uitgevoerd worden.

15. Het maximale aantal te gebruiken dieren is ruim ingeschat, maar wel herleidbaar ten opzichte van de gekozen onderzoeksopzet. De onderzoekers hanteren een goede strategie om ervoor te zorgen dat er met het kleinst mogelijke aantal dieren wordt gewerkt waarmee nog een wetenschappelijk betrouwbaar resultaat kan worden verkregen. Gedragsexperimenten en analyse van hersenen worden uitgevoerd bij dezelfde dieren, waardoor er minder dieren nodig zijn. Indien blijkt dat het intraperitoneaal injecteren zelf geen effect heeft op de gedragstaak, dan wordt deze controlegroep in latere experimenten achterwege gelaten. De onderzoeker verwacht dat er in totaal minder dieren gebruikt zullen worden dan aangevraagd, aangezien de resultaten die gedurende de looptijd van het onderzoek worden gekregen gebruikt worden voor het design van vervolgexperimenten, waarbij sommige experimenten mogelijk niet langer zinvol blijken. De DEC vermoedt dat de aanvrager uiteindelijk veel minder dieren zal gebruiken dan worden aangevraagd en vraagt zich af of het niet voor de hand ligt dat de aanvrager dat op enigerlei wijze verwerkt in het totale aantal dieren waarvoor vergunning wordt gevraagd. De commissie wil graag een advies uitbrengen waarbij de uiteindelijke ethische afweging gebaseerd is op een realistisch aantal dieren. De onzekerheid over het aantal dieren dat gebruikt zal worden bemoeilijkt de ethische afweging. De commissie zou daarom graag een tussentijdse rapportage krijgen en/of betrokken worden bij de keuzes voor de hoofdexperimenten op basis van de uitslagen van de pilots, aangezien die keuzes nu, om begrijpelijke redenen, nog niet helder zijn. Die pilotexperimenten zelf zijn nu op globale wijze beschreven, waardoor er ruimte is om het precieze design van de pilotexperimenten op een later moment te bepalen. De commissie is van mening dat die ruimte belangrijk is, zodat voldoende duidelijk wordt hoe het hoofdexperiment moet worden opgezet en om te kunnen berekenen hoeveel dieren daarvoor nodig zijn. De commissie is van mening dat een groepsgrootte van 20 dieren voor het hoofdexperiment al vrij fors is. Indien grotere groepen noodzakelijk blijken dan zou dit via een amendement, waarover de commissie een advies uitbrengt, aangevraagd moeten worden.

16. Het project is in overeenstemming met de vereiste van de verfijning van dierproeven. Er wordt anesthesie toegepast voor alle handelingen waarbij dit is vereist. De gedragstaak veroorzaakt weinig ongerief voor de dieren en helpt mogelijk om verveling tegen te gaan. De individueel gehuisveste dieren krijgen extra bedding en een schuilgelegenheid. De DEC is ervan overtuigd dat de beschreven proefopzet de meest verfijnde is en dat de dierproeven zo humaan mogelijk worden uitgevoerd.

17. Het betreft geen wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. De aanvrager zal in het hele project alleen gebruik maken van mannelijke dieren. De aanvrager geeft hiervoor de volgende onderbouwing: aangezien is aangetoond dat emotionele prikkeling en stressreacties bij vrouwelijke dieren samenhangen met fasen van de oestruscyclus, neemt de inter-individuele variabiliteit af door alleen mannelijke dieren te gebruiken. Bovendien is de hypothese van deze projectaanvraag gebaseerd op data die bij mannelijke dieren zijn verkregen. De DEC is van mening dat de aanvrager in voldoende mate wetenschappelijk heeft onderbouwd dat het om de doelstellingen met zo min mogelijk dieren te bereiken noodzakelijk is om de proeven met alleen mannelijke dieren uit te voeren. Wanneer effecten van stresshormonen op flexibiliteit van geheugen gevonden worden, zou in een vervolgaanvraag onderzocht kunnen worden in hoeverre de gevonden mechanismen ook van toepassing zijn op vrouwelijke dieren.

19. De dieren zullen in het kader van het project gedood worden. Dit is noodzakelijk om de hersenen te kunnen onderzoeken voor het beantwoorden van bepaalde onderzoeksvragen, en voor controle op de juiste plaatsing van de canules. De gebruikte dodingsmethode staat vermeld in bijlage IV van richtlijn 2010/63/EU.

20. Er worden in deze projectaanvraag geen dieren gedood om niet-wetenschappelijke redenen.

NTS

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

D. Ethische afweging

1. Rechtvaardigt het belang van achterhalen hoe de stresshormonen NA en CORT de flexibiliteit van het geheugen van muizen beïnvloeden, en welke hersencircuits daarbij betrokken zijn het ongerief dat de dieren wordt aangedaan, en is aan alle zorgvuldigheidseisen (3V's) voldaan?

2. Er vindt een matige aantasting van het welzijn en een aantasting van de integriteit van de proefdieren plaats. De doelstellingen kunnen niet zonder dieren behaald worden. De onderzoekers doen al het mogelijke om het lijden van de dieren en het aantal dieren te beperken (beschreven in C9 tot C20).

   Voor de wetenschappelijke gemeenschap (met name neurowetenschappers en cognitieve psychologen) is dit onderzoek van belang omdat het nieuwe kennis zal opleveren over de mechanismen achter flexibiliteit van geheugen, over de betrokken hersengebieden, en de invloed van stresshormonen daarop. Geheugenflexibiliteit speelt bij veel gedrag een rol. Kennis over effecten van stress daarop, kunnen daarom van belang zijn voor keuzes omtrent de opzet van onderzoek waarin geheugenflexibiliteit van belang is. De gedragstaak en het theoretische model kunnen door andere onderzoekers gebruikt worden in hun onderzoek.

   Voor patiënten met PTSS is dit onderzoek indirect en op de lange termijn van belang, omdat het kan leiden tot de ontwikkeling van betere diagnostiek en/of nieuwe behandelingen voor hun aandoeningen (met name niet-adaptieve herinneringen). De huidige behandelingen zijn voor meer dan de helft van PTSS-patiënten niet effectief genoeg. Gerichte behandeling op basis van

mechanistisch inzicht in de effecten van stresshormonen op hersenen en flexibiliteit van geheugen zou kunnen bijdragen aan de ontwikkeling van effectievere behandelingen voor mensen die lijden aan (niet-adaptieve) emotionele herinneringen. Dit kan er toe leiden dat de patiënt weer gezond wordt, dan wel een betere kwaliteit van leven heeft. De resultaten zijn waarschijnlijk ook relevant voor de ontwikkeling van effectievere therapieën voor andere stress-gerelateerde psychische stoornissen, zoals angststoornissen. Kunnen beschikken over adequate behandelingen voor stress-gerelateerde psychische stoornissen is van groot belang voor de samenleving.

3. De DEC is overtuigd van het belang van de doelstellingen: te achterhalen hoe de stresshormonen NA en CORT de flexibiliteit van het geheugen van muizen beïnvloeden, en welke hersencircuits daarbij betrokken zijn. Het uiteindelijke doel is beter begrip van de invloed van stress op geheugenflexibiliteit, zodat die kennis gebruikt kan worden voor de ontwikkeling van behandelingen voor mensen die lijden aan stress-gerelateerde mentale aandoeningen, zoals PTSS en angststoornissen.
   De DEC is van mening dat de belangen van de wetenschappelijke gemeenschap en de patiënten voldoende zwaar wegen om het schaden van de belangen van de proefdieren (om gevrijwaard te blijven van een aantasting van hun welzijn en integriteit) te rechtvaardigen. De commissie is overtuigd van de kwaliteit van het werk van de aanvrager. De DEC is van mening dat het project goed is opgezet, en dat de gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn, dat de gebruikte aanpak de meest verfijnde is en dat zij zal kunnen voorkomen dat mens, dier en het milieu onbedoelde negatieve effecten ondervinden als gevolg van de dierproeven. De onderzoeker verwacht dat er uiteindelijk minder dan het aantal aangevraagde dieren nodig zal zijn om de doelstelling te behalen, omdat er op basis van behaalde resultaten keuzes gemaakt zullen worden in de proefopzet en een selectie van de beschreven experimenten uitgevoerd zal worden. De onzekerheid over het aantal dieren dat gebruikt zal worden bemoeilijkt de ethische afweging. De commissie zou daarom graag een tussentijdse rapportage krijgen en/of betrokken worden bij de keuzes voor de hoofdexperimenten op basis van de uitslagen van de pilots, aangezien die keuzes nu, om begrijpelijke redenen, nog niet helder zijn.
   De DEC is 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

   ☒ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
   - o 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.
   - o Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
   - X Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten: de commissie zou graag een tussentijdse rapportage krijgen en/of betrokken worden bij de keuzes voor de hoofdexperimenten op basis van de uitslagen van de pilots (zie C15).

☐ De DEC adviseert de vergunning niet te verlenen vanwege:

- De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:...
- De volgende doorslaggevende ethische bezwaren:...
- De volgende tekortkomingen in de aanvraag:...

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn geen knelpunten of dilemma's geconstateerd – zowel binnen als buiten de context van het project - die de verantwoordelijkheid en competentie van de DEC overstijgen.

Van: info@zbo-ccd.nl
Verzonden: donderdag 28 juli 2022 13:05
Aan: Postbus instantie voor dierenwelzijn
CC: [redacted] Postbus DierExperimenten Commissie
Onderwerp: 2022-0008-Aanhouden AVD10300202216030

Geachte [redacted]

Op 05-05-2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neural mechanisms underlying stress hormone effects on memory flexibility" met aanvraagnummer AVD10300202216030. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Niet technische samenvatting
De NTS dient voor het brede Nederlandse publiek navolgbaar te zijn. Uw NTS bevat over het algemeen lastig taalgebruik. Kunt u de NTS herzien en aanpassen zodat de tekst navolgbaar is voor het brede publiek?
Kunt u bij het aanpassen van de NTS de term 'laagste diersoort', zoals genoemd onder Vervanging en Verfijning, aanpassen of verduidelijken?

Onder het kopje 'Verfijning' in de NTS noemt u dat beperkte voerverstrekking de gezondheid en levensverwachting van de dieren verhoogt. Kunt u deze stelling onderbouwen?

Onduidelijkheden
U geeft in alle bijlagen dierproeven aan dat u de dieren tijdens het experiment zult onthouden van voer. Kunt u het onthouden van voer en de onderbouwing daarvoor beschrijven onder 'C. Accommodation and care' van de bijlagen dierproeven?

Onder 'E. Humane endpoints' noemt u verschillende symptomen die tot een humaan eindpunt zouden kunnen leiden. Kunt u hierbij aangeven hoe lang de symptomen aan zullen houden voordat een humaan eindpunt bereikt zal worden en met welk interval u de dieren zult monitoren?

Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen omdat de gegevens onvolledig of onduidelijk zijn.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van dit bericht op. U kunt dit aanleveren via NetFTP.

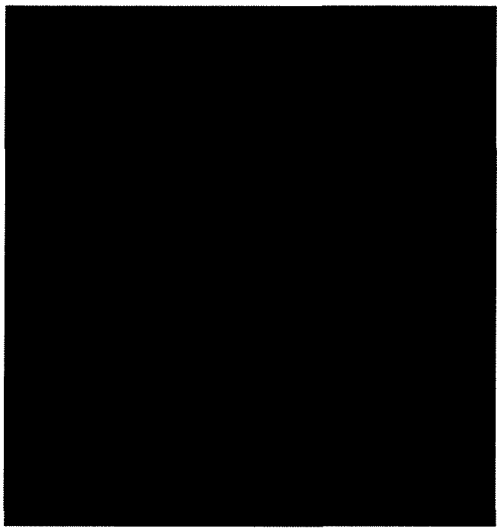
Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

[redacted]
www.centralecommissiedierproeven.nl

.....
Postbus 93118 | 2509 AC | Den Haag
.....



Nijmegen, 8 augustus 2022

Geachte leden van de Centrale Commissie Dierproeven,

Hartelijk dank voor uw reactie op ons project *'Neural mechanisms underlying stress hormone effects on memory flexibility'*, geregistreerd onder AVD10300202216030.

Hieronder beantwoorden wij alle vragen van de commissie. In de bijlage beschrijving dierproeven hebben wij alle nieuwe of herschreven tekst in rood aangegeven. Tevens is de nieuwe of herschreven tekst ook in deze brief rood weergegeven zodat duidelijk wordt naar aanleiding van welke vraag de nieuwe tekst geschreven is. In de NTS konden wij de herziene tekst helaas niet in rood markeren.

Niet technische samenvatting
De NTS dient voor het brede Nederlandse publiek navolgbaar te zijn. Uw NTS bevat over het algemeen lastig taalgebruik. Kunt u de NTS herzien en aanpassen zodat de tekst navolgbaar is voor het brede publiek?
We hebben de NTS herzien om de tekst duidelijk te maken voor een breed publiek.

Kunt u bij het aanpassen van de NTS de term 'laagste diersoort', zoals genoemd onder Vervanging en Verfijning, aanpassen of verduidelijken?
Bij 'Vervanging' hebben we de tekst zodanig herschreven dat het gebruik van de term 'laagste diersoort' niet meer nodig is: *'Eerder onderzoek bij de mens heeft aangetoond dat stress en stresshormonen de flexibiliteit van het geheugen verminderen. Omdat we in dit project een oorzakelijk verband willen aantonen tussen bepaalde hersenmechanismen en stresshormoon effecten op de flexibiliteit van het geheugen is het gebruik van menselijk materiaal niet mogelijk. Daarnaast zijn de gewenste gecontroleerde studies niet uitvoerbaar in mensen. Gezien de complexiteit van het geheugen en de beïnvloeding van het geheugen door stress is het niet mogelijk het onderzoek uit te voeren met dierproefvrije alternatieven. Tevens is het belangrijk om voor de uitvoering van deze experimenten een diersoort te gebruiken waarbij de onderzochte hersenprocessen vergelijkbaar zijn met die van de mens. Daarom kunnen voorgestelde experimenten ook niet worden uitgevoerd met ongewervelde dieren. De muis is een goed model voor het onderzoeken van stresseffecten op het brein en geheugen. De muis biedt bovendien mogelijkheden tot zeer specifieke metingen en manipulaties van hersenprocessen.'* Onder 'Verfijning' hebben we de zin waarin deze term voorkwam geheel verwijderd.

Onder het kopje 'Verfijning' in de NTS noemt u dat beperkte voerverstrekking de gezondheid en levensverwachting van de dieren verhoogt. Kunt u deze stelling onderbouwen?
Diverse studies hebben aangetoond dat beperkte voerverstrekking de gezondheid en levensverwachting van dieren verhoogt. In de bijlagen dierproeven refereren we naar een van deze studies ter onderbouwing van deze stelling. Omdat we in de NTS geen literatuurverwijzingen kunnen opnemen, hebben we hier de tekst zodanig aangepast dat het duidelijk is dat de stelling dat

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beperkte voerverstrekking de gezondheid en levensverwachting van de dieren verhoogt gebaseerd is op resultaten van eerder gedaan onderzoek: *'Onderzoek heeft uitgewezen dat beperkte voerverstrekking niet ongezond is maar juist de gezondheid en levensverwachting van de dieren verhoogt.'*

Onduidelijkheden

U geeft in alle bijlagen dierproeven aan dat u de dieren tijdens het experiment zult onthouden van voer. Kunt u het onthouden van voer en de onderbouwing daarvoor beschrijven onder 'C. Accommodation and care' van de bijlagen dierproeven?

De beperkte voerverstrekking gedurende een deel van het experiment en de onderbouwing daarvoor hebben we nu als volgt beschreven onder 'C. Accommodation and care' van de bijlagen dierproeven: *'All animals will be food restricted (to 90% of their free-feeding body weight) starting with a gradual transition of 2-3 days prior to the conditioning phase of the memory inference task. Food restriction is needed in order to motivate the animals to perform the task and work for a reward. We will feed the animals daily after the learning task. The total duration of food restriction is 8-10 days. This protocol is in accordance with the code of practice from the NCad on food restriction in neurocognitive research (1). We will regularly weigh the animals to keep track of their body weight.'*

Onder 'E. Humane endpoints' noemt u verschillende symptomen die tot een humaan eindpunt zouden kunnen leiden. Kunt u hierbij aangeven hoe lang de symptomen aan zullen houden voordat een humaan eindpunt bereikt zal worden en met welk interval u de dieren zult monitoren?

We geven nu aan dat humane eindpunten vooral gedurende de eerste 2-3 dagen na de operatie kunnen worden bereikt. Daarom zullen de dieren gedurende deze eerste 3 dagen na de operatie dagelijks worden gemonitord: *'Although unlikely (based on previous experience <2%), the first 3 days of the recovery phase after surgery is the most critical period at which humane endpoints are reached. During this period, animals may show reduced food intake and reduced locomotor or grooming behavior. Therefore, animals will be monitored daily until 3 days post-surgery for signs of sickness, infection, excessive weight loss (>15% in 2 days, plus a criterion of 20% overall weight loss), or other signs of diminished well-being.'* Daarnaast zullen de dieren ook gedurende de gedragstest regelmatig worden gecheckt voor humane eindpunten: *'During the experiment, animals will be monitored routinely to check for standard humane endpoints and weighed 2-3 times per week to keep track of their body weight.'*

We hopen de commissie middels deze brief van voldoende informatie te hebben voorzien voor een ethische afweging. Bij voorbaat dank voor uw reactie.

Met vriendelijke groeten,

[redacted]

Van: Info-zbo <info@zbo-ccd.nl>
Verzonden: dinsdag 16 augustus 2022 13:18
Aan: 'info@zbo-ccd.nl'; Postbus instantie voor dierenwelzijn
CC: [redacted]
Onderwerp: RE: Aanhouden AVD10300202216030

Geachte [redacted]

In de onderstaande mail zijn de vragen betreffende de NTS blijven staan. Deze heeft u in de vorige vragenronde al beantwoord en deze vragen mag u negeren. Graag zien we wel een antwoord op de vraag die staat onder onduidelijkheden.
Mijn excuses voor de verwarring.

Met vriendelijke groet,
[redacted]
Namens:
Centrale Commissie Dierproeven

www.centralecommissiedierproeven.nl
.....
Prinses Beatrixlaan 2 | 2595 AL | Den Haag
Postbus 93118 | 2509 AC | Den Haag
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T: 0800-7890789 E: info@zbo-ccd.nl

Van: info@zbo-ccd.nl <info@zbo-ccd.nl>
Verzonden: dinsdag 16 augustus 2022 13:14
Aan: instantievoordierenwelzijn@radboudumc.nl
CC: [redacted]@radboudumc.nl; dierexperimentencommissie@radboudumc.nl
Onderwerp: Aanhouden AVD10300202216030

Geachte [redacted]

Op 05-05-2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neural mechanisms underlying stress hormone effects on memory flexibility" met aanvraagnummer AVD10300202216030. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Niet technische samenvatting
De NTS dient voor het brede Nederlandse publiek navolgbaar te zijn. Uw NTS bevat over het algemeen lastig taalgebruik. Kunt u de NTS herzien en aanpassen zodat de tekst navolgbaar is voor het brede publiek?
Kunt u bij het aanpassen van de NTS de term 'laagste diersoort', zoals genoemd onder Vervanging en Verfijning, aanpassen of verduidelijken?

Onder het kopje 'Verfijning' in de NTS noemt u dat beperkte voerverstrekking de gezondheid en levensverwachting van de dieren verhoogt. Kunt u deze stelling onderbouwen?

Onduidelijkheden
De voor dit onderzoek benodigde intraperitoneale injecties (inclusief het fixeren van de dieren voor die injecties) zijn ook stressvol voor de dieren. De commissie vermoedt dat er in voorgaande jaren

soortgelijke studies door uw onderzoeksgroep gedaan zijn waaruit blijkt hoe lang het effect daarvan aanhoudt (bijvoorbeeld door het meten van bloedwaardes van corticosteron). De commissie had het erg gewaardeerd wanneer u uw mening hierover had onderbouwd met data, met name omdat ook niet duidelijk is aangegeven in de bijlage dierproeven hoe kort de injecties voor de flexibele geheugen tests worden toegediend. In de projectaanvraag is weliswaar een adequaat no-go criterium ingebouwd dat het onderzoek stopt als blijkt dat door die handelingen opgewekte stress inderdaad teveel interfereert met de hoofdexperimenten, maar reeds bestaande kennis daarover zou van belang kunnen zijn voor vroegtijdige optimalisatie van de experimentele omstandigheden. Zou u informatie over bovenstaande kunnen geven?

Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen omdat de gegevens onvolledig of onduidelijk zijn.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van dit bericht op. U kunt dit aanleveren via NetFTP.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

[redacted]
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.....
T: 0800 789 0789
E: info@zbo-ccd.nl

[redacted]

Nijmegen, 18 augustus 2022

Geachte leden van de Centrale Commissie Dierproeven,

Hartelijk dank voor uw verdere vragen op ons project *'Neural mechanisms underlying stress hormone effects on memory flexibility'*, geregistreerd onder AVD10300202216030.

Hieronder beantwoorden wij de vragen van de commissie. In uw reactie stonden nog een aantal vragen over de niet technische samenvatting die wij al op 8 augustus j.l. hadden beantwoord. Uit verdere correspondentie bleek dat wij deze vragen nu konden negeren. In de bijlage beschrijving dierproeven hebben wij de nieuwe tekst in rood aangegeven. Tevens is de nieuwe tekst ook in deze brief rood weergegeven.

Onduidelijkheden

De voor dit onderzoek benodigde intraperitoneale injecties (inclusief het fixeren van de dieren voor die injecties) zijn ook stressvol voor de dieren. De commissie vermoedt dat er in voorgaande jaren soortgelijke studies door uw onderzoeksgroep gedaan zijn waaruit blijkt hoe lang het effect daarvan aanhoudt (bijvoorbeeld door het meten van bloedwaardes van corticosteron). De commissie had het erg gewaardeerd wanneer u uw mening hierover had onderbouwd met data, met name omdat ook niet duidelijk is aangegeven in de bijlage dierproeven hoe kort de injecties voor de flexibele geheugen tests worden toegediend. In de projectaanvraag is weliswaar een adequaat no-go criterium ingebouwd dat het onderzoek stopt als blijkt dat door die handelingen opgewekte stress inderdaad teveel interfereert met de hoofdexperimenten, maar reeds bestaande kennis daarover zou van belang kunnen zijn voor vroegtijdige optimalisatie van de experimentele omstandigheden. Zou u informatie over bovenstaande kunnen geven?

Bedankt voor deze relevante vraag. In de eerdere vragenronde met de DEC is de stress gerelateerd aan IP injecties ook aan de orde gekomen. De essentie is als volgt: Wij hebben het stress effect van IP injecties voorafgaande een leertaak niet eerder bij muizen onderzocht. Voorheen hebben wij vooral met ratten gewerkt. Daar hebben we de injecties altijd subcutaan gegeven en vonden we dat een injectie bij ratten die goed gewend zijn geraakt aan de procedure slechts in zeer beperkte mate tot een stressreactie leidt. Bij muizen hebben wij eerder alleen post-(geheugentest)training IP injecties gegeven en naar latere consequenties op het geheugen gekeken. Bij sommige leertaken vonden we dat de IP injectie weinig of geen verstorende effecten had op het latere geheugen in vergelijking met een niet-injectie controle groep. Bij andere leertaken vonden wij echter dat de post-(geheugentest)training IP injectie wel invloed had en waren wij niet in staat dit effect te verminderen door de gewenningsprocedure aan te passen. Dus de mogelijke consequentie van de stressreactie van een IP injectie lijkt sterk bepaald te worden door de specifieke leertaak waar deze wordt toegepast. Aangezien we de flexibele geheugentaak die we in dit project beschrijven niet eerder hebben gebruikt, zullen we dus de mogelijke effecten van de injectieprocedure op deze specifieke leertaak moeten onderzoeken. Dit is ook de reden dat wij ook in de voorgestelde experimenten niet-injectie groepen meenemen om de effecten van de IP injectieprocedure te kunnen bepalen. Verder staat in DAP1 beschreven dat we in de pilot het

[redacted]

effect van de injectie als stressor willen onderzoeken, en hoe we hier eventueel verder op gaan handelen door bijvoorbeeld de handlingsessies of het tijdstip van de injectie aan te passen. Wat het tijdstip van de injectie betreft: In onze eerder uitgevoerde experimenten met ratten gaven we deze injectie tussen 30 en 60 minuten voor de leertaak. Het precieze tijdstip van de IP injectie voor de voorgestelde experimenten zal geoptimaliseerd moeten worden waar we enerzijds voldoende tijd willen geven zodat eventuele stressreacties door de injectieprocedure kunnen uitdoven maar anderzijds ook rekening moeten houden met de kinetiek (tijdsduur van absorptie en afbraak) van de toegediende stoffen. Het streven is om ook hier de IP injectie tussen 30 en 60 minuten voor de start van de leertaak te geven en hebben dit nu ook toegevoegd aan elke DAP. Voor DAP1 (Stress hormone administration, 2A. Experimental approach and primary outcome parameters): '*The noradrenergic stimulant yohimbine (expected dose range: 0.3 - 3.0 mg/kg) or its saline vehicle, or CORT (expected dose range: 1 - 10 mg/kg) or its vehicle (5% ethanol in saline) will be administered by i.p. injection shortly (likely between 30 and 60 min) before the memory inference test.*'

We hopen de commissie middels deze brief van voldoende informatie te hebben voorzien voor een ethische afweging. Bij voorbaat dank voor uw reactie.

Met vriendelijke groeten,

[redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 93118 2509 AC Den Haag

Stichting Katholieke Universiteit Nijmegen
[redacted]
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6500 HB NIJMEGEN

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Onze referentie
Aanvraagnummer
AVD10300202216030
Bijlagen
3

Datum 19 augustus 2022
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 5 mei 2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neural mechanisms underlying stress hormone effects on memory flexibility" met aanvraagnummer AVD10300202216030. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed. Uit artikel 10a, eerste lid van de Wet op de dierproeven (hierna: de wet) volgt daarom dat het is toegestaan om uw project uit te voeren binnen de gestelde vergunningsperiode. Deze vergunning wordt afgegeven voor de periode van 19 augustus 2022 tot en met 30 juni 2027.

De onderbouwing van deze beslissing vindt u onder 'Overwegingen'.

Procedure

Advies dierexperimentencommissie
Wij hebben advies gevraagd bij de dierexperimentencommissie RU DEC (hierna: DEC). Dit advies is ontvangen op 25 juli 2022. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, derde lid van de wet.

Nadere vragen aanvrager
Op 28 juli 2022 en 16 augustus 2022 hebben wij u om aanvullingen gevraagd. U heeft tijdig antwoord gegeven. Het verzoek om aanvullingen had betrekking op de huisvesting, de intraperitoneale injecties, de humane eindpunten en de Niet-technische Samenvatting. Uw reactie is betrokken bij de behandeling van uw aanvraag.

Overwegingen
Wij kunnen ons niet geheel vinden in de inhoud van het advies van de DEC. De CCD ziet af van de door de DEC voorgestelde aanvullende voorwaarde om de uitkomsten van de pilotexperimenten terug te koppelen aan de DEC.

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 93118, 2509 AC 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. Nadat u een bezwaarschrift heeft ingediend kunt u een voorlopige voorziening vragen bij de voorzieningenrechter van de rechtbank in de vestigingsplaats van de vergunninghouder. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisende situatie.

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 vergunninghouder valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl, stuur een e-mail naar info@zbo-ccd.nl of neem telefonisch contact met ons op: 0800 789 0789.

Datum:
19 augustus 2022
Aanvraagnummer:
AVD103002022160 30

Centrale Commissie Dierproeven
namens deze:

[redacted]

Bijlagen:
- Projectvergunning
- DEC-advies
- Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

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

deze projectvergunning voor het tijdvak 19 augustus 2022 tot en met 30 juni 2027, voor het project "Neural mechanisms underlying stress hormone effects on memory flexibility" met aanvraagnummer AVD10300202216030, na advies van dierexperimentencommissie RU DEC. De functie van de verantwoordelijk onderzoeker is Postdoc [redacted] Het besluit is gebaseerd op de volgende (aangepaste) stukken:

1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 5 mei 2022
2 de bij het aanvraagformulier behorende bijlagen:
   a Projectvoorstel, zoals ontvangen op 25 juli 2022;
   b Bijlagen dierproeven
      - 3.4.3.1 Determining stress hormone effects on memory flexibility, zoals ontvangen op 18 augustus 2022;
      - 3.4.3.2 Determining the effect of local administration of stress hormones into specific brain areas on memory flexibility, zoals ontvangen op 18 augustus 2022;
      - 3.4.3.3 Providing causal evidence for the modulation of neural systems in regulating stress hormone effects on memory flexibility, zoals ontvangen op 18 augustus 2022;
   c Niet-technische Samenvatting van het project, zoals ontvangen op 8 augustus 2022;
   d Advies van dierexperimentencommissie, zoals ontvangen op 25 juli 2022
   e De aanvullingen op uw aanvraag, zoals ontvangen op 8 augustus 2022, 18 augustus 2022.

Naam proef	Diersoort/ Stam	Aantal dieren	Ongerief
3.4.3.1 Determining stress hormone effects on memory flexibility			
	Muizen (Mus musculus) / C57BL/6J	510	100,0% Matig
3.4.3.2 Determining the effect of local administration of stress hormones into specific brain areas on memory flexibility			
	Muizen (Mus musculus) / C57BL/6J	2.040	100,0% Matig
3.4.3.3 Providing causal evidence for the modulation of neural systems in regulating stress hormone effects on memory flexibility			
	Muizen (Mus musculus) / C57BL/6J	3.350	100,0% Matig

Aanvraagnummer: AVD10300202216030

Geldende voorschriften

Wij wijzen u op onderstaande geldende voorschriften, die volgen uit artikel 1d, vierde lid, artikel 10, eerste lid en/of artikel 10a3 van de wet.

- Go/ no go momenten worden voor aanvang van elk experiment afgestemd met de IvD.
- Het is verboden 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.
- Het is verboden dierproeven te verrichten voor een doel waarvan het belang niet opweegt tegen het ongerief dat aan het proefdier wordt berokkend.
- Overige wettelijke bepalingen blijven van kracht.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD10300202216030

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, derde lid van de wet. Uit artikel 10b, eerste lid van de wet 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, eerste lid van de wet de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven. Artikel 10b, tweede en derde lid van de wet schrijven 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 van de wet 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 van de wet moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13c van de wet volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13d van de wet 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 NTS van dit project staat gepubliceerd op de website van de EU:
https://webgate.ec.europa.eu/envdataportal/web/resources/alures/submission/nts/list
U kunt de NTS het makkelijkste vinden door op de Nederlandse titel te zoeken. De samenvattingen op deze website hebben een Europees volgnummer welke verschilt van het Nederlandse NTS-nummer. Voor meer informatie over de NTS en een lijst met zowel de Nederlandse als Europese volgnummers gaat u naar deze pagina op de website van de CCD:
https://www.centralecommissiedierproeven.nl/onderwerpen/themas/niet-technische-samenvattingen

The NTS of this project is published on the website of the EU and can be found here:
https://webgate.ec.europa.eu/envdataportal/web/resources/alures/submission/nts/list
The easiest way to find the NTS is to search on the Dutch title. The summaries on this website are indicated with their European serial number, which is different from the Dutch NTS number.
You can find more information about the NTS and a list with the Dutch NTS numbers and their corresponding European counterparts on the website of the CCD:
https://www.centralecommissiedierproeven.nl/onderwerpen/themas/niet-technische-samenvattingen