	Inventaris Wob-verzoek W15-11								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 2015126								
1	Aanvraagformulier				x		x	x	
2	Niet-technische samenvatting	x							
3	Projectvoorstel				x		x	x	
4	Bijlage beschrijving dierproeven 1			x					
5	Bijlage beschrijving dierproeven 2			x					
6	Bijlage beschrijving dierproeven 3			x					
7	Bijlage beschrijving dierproeven 4			x					
8	Bijlage beschrijving dierproeven 5			x					
9	Flow chart				x		x	x	
10	Overzicht aantallen				x		x	x	
11	DEC-advies				x		x	x	
12	Ontvangstbevestiging				x		x	x	
13	Mail indienen 15-7-2015				x		x	x	
14	Mail aanvraag 20-7-2015				x		x	x	
15	Mail vervolgbrief 31-7-2015				x		x	x	
16	Vervolgbrief				x		x	x	
17	Acceptatiebrief				x		x	x	
18	Mail aanvullende informatie 10-8-2015				x		x	x	
19	Brief aanvullende informatie				x		x	x	
20	Beschikking				x		x	x	
21	Vergunning			x					
22	Mail beschikking 13-8-2015				x		x	x	
23	Advies CCD		x						x

16 JULI 2015

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☑ Ja > *Vul uw deelnemernummer in* 80101 Nederlands Herseninstituut-KNAW 1126

☐ Nee > *U kunt geen aanvraag doen*

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

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

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

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

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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

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

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

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

1.7 Is er voor deze projectaanvraag een gemachtigde?

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

☑ Nee

2 Over uw aanvraag

2.1 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 3

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

Vul uw vergunde projectnummer in en ga verder met vraag 2.2

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

Vul uw vergunde projectnummer in en ga verder met vraag 2.3

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

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

☑ Nee > Ga verder met vraag 3

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

☑ Nee > Ga verder met vraag 3

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

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?

Startdatum	0 1 - 0 8 - 2 0 1 5
Einddatum	0 1 - 0 8 - 2 0 2 0

3.2 Wat is de titel van het project?

Neurobiology of compulsive behavior and its components: Brain stimulation and ...

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

Compulsief gedrag en zijn componenten: neurobiologische metingen en hersenstimulatie

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

Naam DEC	DEC-KNAW
Postadres	[redacted] Amsterdam
E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

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

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

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

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

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

Overige bijlagen, indien van toepassing

- [ ] Melding Machtiging
- [x] Appendixen 5 maal; flow chart

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	[redacted]
Functie	Directeur Instituten KNAW
Plaats	Amsterdam
Datum	10-07-2015
Handtekening	[redacted]

Centrale Commissie Dierproeven

Form
Project proposal

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101 – Nederlands Herseninstituut-KNAW
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW
1.3	Provide the title of the project.	Neurobiology of compulsive behavior and its components: Brain stimulation and measurements

2 Categories

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

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

3 General description of the project

3.1 Background

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

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

The term compulsivity is used to describe dysfunctional behavior in various neuro-psychiatric disorders such as substance and behavioral addictions, obsessive-compulsive disorder (OCD), impulse-control disorders, and eating disorders. The presence of compulsive tendencies in all of these disorders makes a

strong case for a shared underlying mechanism. Compulsive behavior is characterized by the feeling that one 'has to' perform a specific act as a result of an urge (e.g., to avoid a negative emotional state). At the same time, patients are aware of the conflict between the performed act and maintaining their quality of life. Thus, compulsivity produces behavior that we perform against our will and despite its negative consequences. Examples of such consequences are the OCD patient unable to hold a job because he/she spends almost every waking hour cleaning his/her house or the addict that isolates him/her-self from friends and family because he/she continues to relapse to drug abuse despite the best intentions not to. It has been suggested that compulsivity is a so-called 'endophenotype', a behavioral pattern or response mode that is preserved across different disorders. In order to better characterize this concept, its composition can be described as separate components: 1) persistence of performance, 2) elicitation of undesirable consequences, 3) escalation of symptoms over time, 4) aggravation by stress and anxiety, 5) exaggerated habit formation, 6) response inflexibility, and 7) loss of voluntary control. Whereas 1), 2), 3) and 7) are components that cannot be studied separately from the compulsive phenotype itself, 4), 5) and 6) may be endophenotypes that exist in the normal population and lower the threshold for compulsive behavior to develop. **For a better understanding of disorders featuring compulsivity, both the obvious pathological phenotype (compulsivity) itself, as well as these individual components have to be investigated**.

One prominent theory of compulsive behavior proposes a dysfunction or imbalance between competing brain systems: the system that controls more purposeful, goal-directed behavior and the system that supports automatic, habitual behavior (Everitt and Robbins, 2005; Dalley et al, 2011). Goal-directed behavior is based on knowledge of the causal relationship between an action and its consequences (outcomes), and is only performed when those consequences are desired. In contrast, habitual behavior consists of actions that are automatically triggered by environmental stimuli regardless of the current desirability of the consequences, insensitive to its outcomes. These stimulus-response associations that mediate habitual behavior have been strengthened either by reward (positive reinforcement; e.g., drug abuse that improves the user's mood) or by the omission of an aversive event (negative reinforcement; e.g., house cleaning in order to avoid feeling anxious). Habitual behavior can arise under many conditions, the most common one of which is extensive behavioral repetition (Dickinson et al, 1985). However, habits can also arise from failures in goal-directed control and exposure to stress, which can lead to habitual behavior even without extensive behavioral repetition, underlining the competing hierarchy between these behavioral control systems. Therefore, **both systems, habit and goal-directed, can contribute to the likelihood that a habit will be formed in a given situation**.

In patients suffering from psychiatric disorders with compulsivity, such as OCD and drug addiction, an increased tendency towards forming habits has been reported (i.e., in these individuals habits form faster and the habit system dominates their behavior; Voon et al, 2014; Gillan et al, 2015), which has been observed regardless of whether such habits work toward gaining reward or toward avoiding punishment. Stress and anxiety, states present in many disorders, may contribute to this enhanced habit formation (Schwabe et al, 2011). Moreover, it has been suggested that in such a situation habits can become excessive and progressively compulsive as a result of disturbances of brain systems controlling behavior (e.g., a drug-use habit escalates into compulsive drug addiction or cleanliness escalates into obsessive-compulsive cleaning (escalation)). However, how far aberrant habit formation contributes to compulsivity is currently still unclear. Also unclear is whether hypo-function of the goal-directed or hyper-function of the habitual system drives the exaggerated tendency to display habits, and the extent of stress and anxiety as driving forces. Importantly, **despite the habit-hypothesis being the most prominent contemporary theory, dysfunction of any or all of the other individual components mentioned above** (e.g., response inflexibility or insensitivity to negative consequences etc.) **are alternative sources of compulsive behavior** that may or may not interact with habits to produce pathology. Thus, for a better understanding of disorders featuring compulsivity and in order to answer the following questions, both the compulsive behavior itself ('models') as well as all individual components potentially contributing to compulsivity and their interaction have to be investigated. How are these components related to each other? Are habit-prone individuals more susceptible to develop compulsivity? Do habits escalate into compulsivity? Is compulsivity an "endophenotype" that is linked to genetic variations in neurobiological processes? Or are these patients just unable to flexibly change or lose voluntary control of their behavior? And importantly: What are the underlying neurobiological mechanisms of these processes?

The basal ganglia, a subcortical brain system found in all mammals, are thought to serve the purpose of selecting a behavior strategy appropriate for a given situation through interaction with cortical regions that participate in higher cognitive processes and sensorimotor performance. The main input nucleus of

the basal ganglia, the striatum, receives inputs from different functional units of the cortex, and the neurotransmitter dopamine acts as a regulator of cortical information flow through the striatum. Several regions of the cortex and the striatum are thought to be involved in controlling goal-directed and habitual behavior. For example, the medial striatum and the medial prefrontal cortex (mPFC) have been shown to subserve learning involving goal-directed behavior. In contrast, the dorsolateral striatum (DLS) and the infralimbic cortex (IFC) are necessary for the formation of habits. Dysfunction of these systems is indicated in several compulsive disorders: 1) Drug addiction and OCD are characterized by altered activity of the dopamine system, which could manifest as a tendency for natural rewards to lose their value. 2) Dysfunctions of the striatum and mPFC are present in addiction, OCD, obesity, and bulimia nervosa (Robbins et al, 2011). In summary, **different 'cortico-striatal loops' are putatively associated with aspects of compulsivity** but it is still unclear which specific brain systems mediate this development and how they interact.

Although a variety of behavioral and pharmacological treatments for compulsive disorders are available, in many cases such therapy is not successful. Thus, it is of great importance to identify new targets and to develop more effective therapies. A promising avenue opened up when it became clear that **deep-brain stimulation (DBS) is effective in a number of psychiatric disorders**. Although it is not exactly clear how this high-frequency electrical stimulation exerts its effects, recent data suggests that it may interrupt the pathological activity of a cortico-striatal loop, allowing an attentional shift away from excessive processing of disease-related, habit/compulsivity-eliciting stimuli and restoration of goal-directed behavior. Thus, stimulation of a relatively small target area may potentially lead to rapid, broad, and clinically relevant changes in brain network function. However, as said, our understanding of the mechanisms of action of how DBS reduces compulsivity is still limited. What is the neurobiological basis of the therapeutic effects of high-frequency stimulation in this spectrum of compulsive psychiatric disorders? What are the best and safest brain targets for changing pathological behavior in OCD, addiction, and eating disorders? Thus, another long-term goal of our research line is to contribute to answering such questions. As these disorders are characterized by profound behavioral alterations due to disturbances of affect, motivation and cognition, our research proposed in this application is focused on neurobiological substrates of affect, motivation and cognition in general as well as specific key features (components; see above) of these disorders, such as for example the nature of inflexible behavior.

The main objective of the project is to provide a better understanding of the neurobiology of compulsive behavior. In order to do so, compulsive behavior itself AND a variety of motivated behaviors thought to constitute or contribute to compulsivity (components) will be studied in rodents while neural measurements and interventions are performed.

How to study compulsive behavior in rodents: It is difficult to model all aspects of a psychiatric (human) disorder in animals, but it is feasible to study certain aspects of such psychiatric disorders, focusing on distinct neurobehavioral domains. Human compulsive behavior has many aspects and it is still not clear whether it is a "single endophenotype" with a single underlying mechanism, or whether different forms of compulsivity with different mechanisms exist. Thus, to properly model compulsivity in animals, the use of several models is necessary. Animal models for compulsive disorders can for example model the progression from recreational to compulsive consumption of drugs of abuse or high-fat/high-sugar foods. Furthermore, the development of excessive repetitive performance of purposeless actions, akin to OCD, can for example be studied in the Sapap3-mutant mouse model, which shows excessive grooming behavior associated with increased levels of anxiety (Welch et al, 2007). In a more recent model, mice develop increased grooming behavior after optogenetic stimulation of a corticostriatal brain circuit (Ahmari et al, 2013). Another, pharmacological paradigm allows the study of the development of compulsive licking and/or checking behavior in rats, following repeated administration of quinpirole, a dopamine receptor agonist (Eagle et al, 2014). Finally, compulsive drinking may develop in animals exposed to repetitive timed food presentation (schedule-induced polydipsia (Falk, 1961). Common read-outs of these compulsive behaviors are the registration of natural repetitive actions (grooming, licking). Additional tests may be used to probe the presence of perseverative, inflexible and/or habit-like behavior in operant tasks where an animal may obtain a reward or may avoid a punishment. A special form of this is the signal attenuation model, in which rats are tested in a condition of diminished response feedback (Joel, 2006). In this way, the relation between excessive repetition of natural behaviors likely to establish habits and the presence of compulsivity in more complex, acquired behaviors is studied (including for example cognitive flexibility). At the same time, these models are perfectly suited to study the underlying neuronal mechanisms and test novel treatment strategies. In order to answer the question whether compulsivity is a single 'endophenotype', a behavioral response mode that is preserved across environmental contexts and disorders, it is necessary to use a number of different animal models of

compulsivity. Verification of whether this trait is stable across different behavioral tests and whether the same underlying brain mechanisms can be identified in these tests, will enable the exploration of this question. We hypothesize that there is indeed a single compulsivity trait.

Our group has gathered extensive experience in using various models of compulsive behavior, most notably with compulsive self-administration of cocaine in rats, compulsive grooming in Sapap3-mutant mice, compulsive checking in quinpirole-treated rats, and compulsive responding following "signal attenuation" in mice. In our lab, operant boxes, various mazes, and other test arenas are successfully employed to analyze spontaneous and learned behavior, including habit formation, reversal learning and other tests of cognitive flexibility. These rodent models are routinely combined with *intervention techniques*, such as optogenetic intervention, DBS, intracerebral and systemic drug administration, and *measurements of brain activity,* such as neurochemical and neurophysiological methods including microdialysis, fast-scan cyclic voltammetry, and electrophysiology. Our results showed for example, that dopamine release in the brain is different between rats progressing to compulsive cocaine self-administration and rats that do not. We also found that differences in dopamine signaling in the striatum are predictive for the ability to rapidly learn a reversal of response-reward relations. Furthermore, neuronal activity measured in the striatum and connected cortical regions was predictive of compulsive behavior after repeated quinpirole administration. Based on this experience, we feel that we can take the next step in our research and try to answer the question which common neurobiological mechanisms underlie the development, escalation, and persistence of different forms of compulsive behavior and which brain stimulation targets may contribute to remission of compulsive behavior. To our knowledge, this approach is unique – worldwide, most groups work on a single model of compulsivity, most often substance addiction. Moreover, attempts to reverse compulsive behavior using DBS are still scarce and, if performed, are not followed up by studies of the underlying mechanisms. We are among the few groups worldwide where direct and extensive interactions between animal and clinical scientists is practiced.

Ahmari et al (2013) Repeated cortico-striatal stimulation generates persistent OCD-like behavior. Science, 340(6137), 1234-1239.
Dalley et al (2011) Impulsivity, compulsivity, and top-down cognitive control. Neuron 69, 680–694.
Dickinson (1985) Actions and habits: The development of behavioral autonomy. Philos Trans R Soc Lond B Biol Sci 308:67–78.
Eagle et al (2014) The dopamine D2/D3 agonist quinpirole increases checking-like behavior: a novel possible model of OCD. Behav Brain Res. 264:207-29.
Everitt and Robbins (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat. Neurosci. 8, 1481–1489.
Falk (1961) Production of polydipsia in normal rats by an intermittent food schedule. Science 133, 195-196.
Gillan et al (2015) Functional Neuroimaging of Avoidance Habits in Obsessive-Compulsive Disorder. Am J Psychiatry 172:3.
Marsh et al (2009) Deficient activity in the neural systems that mediate self-regulatory control in bulimia nervosa. Arch. Gen. Psychiatry 66, 51–63.
Robbins (2012) Neurocognitive endophenotypes of impulsivity and compulsivity: towards dimensional psychiatry. Trends in Cognitive Sciences, Vol.16, No.1.
Schwabe et al (2011) Stress, habits and drug addiction: a psychoneuroendocrinological perspective. Exp. Clin. Psychopharm. 19, 53–63.
Voon et al (2014) Disorders of compulsivity: a common bias towards learning habits. Mol Psychiatry 20(3):345-52.
Welch et al (2007) Cortico-striatal synaptic defects and OCD-like behaviors in Sapap3-mutant mice. Nature Aug 23;448 (7156):894-900.

3.2 Purpose

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

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

As explained above, compulsive behavior is believed to be a central common denominator to several neuro-psychiatric disorders such as addictions, obsessive-compulsive disorder (OCD), and eating disorders. Compulsive behavior is probably constituted by a number of individual components, such as behavioral inflexibility, and it is aggravated by stress and anxiety, whereby it is hypothesized that

aberrant habit formation is crucial for its development. The main objective of the project is to provide a better understanding of the neurobiology of compulsive behavior. In order to do so, compulsive behavior itself and a variety of its presumed components will be studied while neural measurements and interventions are performed.

The general research questions addressed by this approach are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

These objectives are achievable because...
1) … the lab has powerful research tools at its disposal (e.g., opto- and chemogenetics, electrophysiological and electrochemical detection of brain activity, animal models for compulsive disorders),
2) … the lab is situated in an excellent research environment with state-of-the-art facilities and close proximity to outstanding scientists at [redacted],
3) … the lab has outstanding national and international collaborators (e.g., [redacted]),
3) … the proposed strategy is heavily grounded on our laboratory's expertise,
4) … technical skills necessary are almost all already in place,
5) … we have a close collaboration with [redacted] warranting proximity to the clinical condition
6) … we have a proven track record of successfully conducting and publishing comparable research in highly respectable scientific journals in the past (e.g., [redacted]).
7) … we have a proven track record of successful grant applications (e.g., [redacted])

References:

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

3.3 Relevance

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

Scientific relevance
The identification, characterization, and understanding of brain pathways mediating a progression from learning a new behavior to its automatic execution and in some cases to pathological compulsive execution, will have great impact on several fields of neuroscience. Such pathways, although assumed for decades, have yet to be verified. All fields that focus on subjects related to the translation of motivation into behavioral action will be affected, including research either restricted to motor systems only or fields exclusively focused on motivation and emotion. Furthermore, this application is aimed at further elucidating a long-standing question in the field - if and how habits, compulsions, behavioral flexibility, stress, anxiety and impulsivity are related to each other. Thus, discovery of such brain pathways and their functional relevance will potentially offer fundamental insights into how we/our brains control behavior.

Social relevance
Compulsivity research is expected to lead to novel insights that may tie psychiatric diseases together that are now often studied and treated separately, and paving the way to define and treat conditions that

are common to obsessive-compulsive spectrum disorders, substance addiction and eating disorders. *Knowledge and understanding of the neurobiological mechanisms of conditions such as exaggerated habitual responding, inflexible behavior or high sensitivity to stress will lead to a better understanding and hopefully an improved acceptance of psychiatric patients in our society.* This knowledge will also be applied in clinical psychiatric research to improve and extend treatment options for individual patients and for the society as a whole. DBS is one of these treatment options, as its application has been successfully extended from neurological disorders to mental health conditions, a relatively new field that still needs improvement. Thus, our results could be of great benefit for psychiatric conditions by identifying new target brain regions for DBS electrode placement to improve therapeutic efficiency (e.g., specific anatomical circuitry underlying maladaptive habits). The lab's close ties with the [redacted] favors the realization of such future DBS applications. For example, if we are able to identify a specific brain pathway that is crucial for rendering inflexible behavior flexible, DBS electrodes could be directed at that region in OCD patients, addicts, and other patients in a clinical trial. Furthermore, stimulation parameters could be optimized in animal models of compulsive disorders. Together, these approaches may improve the quality of life of many different patient populations. Finally, technological advances such as DBS electrodes that can record brain activity in addition to provide stimulation may lead to closed loop, feedback-based appliances, that cannot be developed and applied without thorough testing in experimental animals.

Translational relevance
Our findings will be communicated to basic scientists, clinicians, clinical researchers, and the general public. We will take advantage of the [redacted], operated by professional journalists. We have previously utilized these services for one of my publications in [redacted] in 2014 in order to engage the general public through Dutch media nationwide (e.g., [redacted]). Such outreach is of importance to raise awareness for the problems of patient suffering from compulsivity, but also to support patients by explaining the biological foundation of their troubles to them.
Although the research proposed in this application is fundamental in nature, the knowledge gained from these studies has great potential for translational utilization. An important contributing factor to enable this is the embedding of my [redacted] research group in a larger clinical research team at the [redacted], where we are involved in weekly meetings. These close ties provide optimal conditions to set up translational, multidisciplinary research.

3.4 Research strategy

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

We are interested in how the brain controls behavior. The ultimate, long-term goal is to better understand how processes mediating learning, emotion, and motivation are affected in pathological conditions such as compulsive behavioral disorders and to invigorate the development of new treatment strategies and optimize existing therapies. We have strong ties with clinical researchers studying compulsive behavior, its neurobiological characteristics and its therapies in patients (in particular DBS). To contribute to the understanding of such pathological processes, we use rodent models of compulsive behavior. By choosing a variety of models we aim to discover the shared mechanism in the various presentations of compulsive behavior. To fulfil this aim, we chose several models that reflect sufficiently different phenotypical presentations: Pharmacological (e.g. repeated administration of the dopamine agonist quinpirole leads to compulsive checking and loss of flexible responding), genetic (e.g. Sapap3-mutant mice show increased anxiety and develop compulsive grooming) and addiction models (e.g. extended exposure to self-administration of cocaine leads to compulsive seeking and taking of the drug). Together, they model compulsive behavior from simple repetitive actions (grooming; reminiscent of autism or impulse disorders) to complex behavior (checking and drug seeking; reminiscent of obsessions and substance addiction). The use of mice in addition to rats is based on the fact that transgenic mice such as the Sapap3-mutant mice are up to now the only model with spontaneous compulsive behavior, where no additional pharmacological treatment or behavioral training is required. These are also the models that we have been using for 2-6 years now and, therefore, form our first tier of models, but these models may be extended or replaced by other pharmacological, behavioral and addiction models, that e.g. show a less variable presentation.

These models will be used to study the development of compulsivity, the individual variation of the phenotypes and the behavioral nature of the dysregulation in the first part of the project (**3.4.4.1 Establishing and characterizing rodent behavior that models compulsive behavior and its**

components; see flow chart – the main read-out is behavior). Compulsivity in patients is regarded as behavior performed despite negative consequences. The animal models should incorporate similar negative consequences (e.g. the skin lesions developing in Sapap3-mutant mice as a result of excessive grooming or the foot shocks cocaine-addicted animals are prepared to accept to obtain their drug reward). Furthermore, we are interested in the presence of the various components of compulsivity, such as increased habit formation, diminished cognitive flexibility, and anxiety and aggravation by stress. These components are important as they may represent "building blocks" of a general compulsive phenotype. One of the outstanding questions is whether they are present before compulsivity develops, or whether they may be consequences of that. Our hypothesis is that they are present as predisposing factors that lower the threshold for the development of compulsive activities. In parallel experiments, our clinical collaborators also study the relation between components such as habit formation and overall OCD symptoms. Thus, similar approaches allow translational comparisons. Together, in 3.4.4.1 we will improve behavioral tests but also use these tests to answer questions such as how does compulsive behavior develop and what is the relation with separate behavioral components?

In the subsequent parts of the project, we will focus on the study of the neurobiological mechanisms underlying the behavior (**3.4.4.2 Identification of brain correlates of compulsive behavior and its components**; see flow chart). Here, both (1) normal function of brain circuits responsible for the production of functional behavior, as well as (2) the nature of dysregulation in the "diseased" state have to be investigated. We aim to do so by employing a variety of techniques to measure brain activity and neurotransmitter release in awake rodents. Our prime interest here is to characterize the functional status of the cortico-basal ganglia-thalamic circuits that support normal motivated behavior and are thought to be dysregulated in the case of compulsive behavior. These measurements will allow us to detect neurobiological correlates of compulsive actions (e.g., grooming bouts in transgenic mice; compulsive lever-pressing for drug-related cues in rats).
Results will be discussed with our clinical colleagues and compared with the clinical results that are obtained using various measuring techniques.

In parallel, to discover causal relationships between brain activity and behavior, brain activity is manipulated (**3.4.4.3 Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation**; see flow chart) using different interventions such as DBS, pharmacogenetics, optogenetics, lesions, and pharmacological treatments (the main read-out is behavior). The target position will be based on the knowledge we have of the functional circuits supporting the compulsivity and its components and in normal animals.

Finally, the interplay of neurobiological variables (e.g., neurotransmitters) and neuroanatomical pathways are investigated during behavior (**3.4.4.4 Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation** – main read-out is the relation between behavior and neuronal activity) but also in the anesthetized animal or brain slices, where, in isolation, brain activity is easier to assess and brain stimulation is applied easier (**3.4.4.5 Identification and characterization of neuroanatomical connections and their regulation**; see flow chart – the main read-out is neuronal activity).
Of these two possibilities, the combination with behavior may be considered the highest level of integration. These studies are restricted to the situation where an intervention has resulted in a promising behavioral effect (e.g. anticompulsive effect in one or more of the models) and information on the neurobiological mechanisms of action is wanted.

The experiments under anesthesia (terminal studies) may be applied to obtain information on circuit connections, selectivity of targeted interventions, etc. Thus, the functional status of the corticostriatal pathway in Sapap3-mutant mice may be studied by electrical or more selective optogenetic stimulation of prefrontal areas and electrophysiological recording or Ca-imaging of striatal activity; The choice for such experiments under anesthesia is also driven by the wish to limit discomfort and only use awake animals in experiments that involve behavior or measurements that are known to be fundamentally affected under anesthesia.

Choices and Go/NoGo decisions.
The choice for the first three animal models for compulsive behavior (first tier) is based on their different characteristics that will allow us to discover the shared mechanisms in the various presentations of compulsive behavior. Further model choices in the course of the project will depend on scientific, practical, and animal-welfare factors. The choice is always determined by the wish to have the best practical option to answer our main scientific questions, with the least discomfort for the animals. Once a

behavioral strategy is established and the desired behavior is reproducibly detected (3.4.4.1), brain activity during this behavior is assessed in another set of animals (measurement; 3.4.4.2) or interfered with in another set of animals (intervention; 3.4.4.3) in order to unravel the neurobiological underpinnings of this behavior (GO point). If we are not able to obtain reproducible and validated compulsive behavior (3.4.4.1) such neurobiological measurements (3.4.4.2) or interventions (3.4.4.3) will not be attempted (NOGO point). If we yield promising results, a combination of the two will be attempted (optional GO point; 3.4.4.4), otherwise not (NOGO point). Thus, in summary, we will only start using an animal model in 3.4.4.2 and 3.4.4.3 after this model has been implemented successfully under 3.4.4.1, and we will only start 3.4.4.4 following successful execution of 3.4.4.2 or 3.4.4.3.
An additional approach is the combination of neurobiological measurements and interventions in the anesthetized animal or in brain slices (3.4.4.5). Here, circuits or targets that are of interest are directly studied without preceding behavioral studies. In case of a positive outcome, these targets may be used in the combined studies of 3.4.4.2 and 3.4.4.3, and may eventually be followed by 3.4.4.4.

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

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

3.4.4.1 Establishing and characterizing rodent behavior that models compulsive behavior and its components

Behavioral assays are carried out in standard operant boxes (e.g., levers that can be pressed by the animal to achieve an outcome), touch-screen boxes (touch screens instead of levers), open-field boxes (empty arenas for animals to explore), and mazes of different shapes (more complex arenas animals can explore and find rewards). In these rodent assays (1) models of compulsive behavior; and (2) components of compulsive behavior, among which habit formation, cognitive flexibility, the relation with fear and anxiety and the impact of stress or enrichment are assessed. Assays that will be employed will probe spontaneous behavior, reward-driven behavior, and also punishment-driven behavior. Depending on the model and the additional component(s) studied, the total testing time for individual animals will vary between 2 and 6 months, but transgenic animals spontaneously showing compulsive behavior may be followed for up to 1 year. Important additional (e.g. correlational) information is expected from testing animals in more than one behavioral assay, one of which is assessing the compulsive behavior. Thus, e.g. anxiety and reward-driven behavior may be characterized before an animal is made compulsive by repeated quinpirole administration; cognitive flexibility is tested in an animal before and/or after being trained to compulsively self-administer cocaine; a Sapap3-mutant mouse that shows compulsive grooming is studied in a habit-formation paradigm, etc.

The general organization is:
A) to establish and validate animal *models* for compulsive behavior – in pilot experiments a satisfactory paradigm is selected for further studies.
B) to characterize the tested population of animals (mice and rats) for individual differences to unravel biological mechanisms.
C) to establish and validate behavioral methods to assess *components* of compulsive behavior – in pilots a satisfactory paradigm is selected.
D) to characterize the compulsivity in relation to its behavioral components – based on the paradigms developed in C, experiments in which one of the components is tested in one of the models of compulsive behavior. All these experiments will also include the testing of general anxiety as a second component tested.

We will start in A) with 3 models (Sapap3-mutant, cocaine self-administration and quinpirole treatment) and characterize individual differences in B). We will start in C) with habit formation and combine that in D) with the three models.

Aspects of this section that will extend beyond pure behavioral testing are:
a. Drug self-administration will require catheter implantation in order to apply intravenous drug injection.
b. Tests to establish "true" compulsivity in rewarded behavior will involve testing behavior in combination

with foot shocks and acquired taste aversion.
c. Sapap3 mutant mice can develop a phenotype with constitutional discomfort. Close monitoring prevents development of skin lesions with more than moderate discomfort.
d. Impact of acute or chronic stress on behavior and well-being will be monitored by measurement of plasma levels of e.g. corticosterone.

3.4.4.2 Identification of brain correlates of compulsive behavior and its components

Once a behavioral strategy is established and the desired behavior is detected, brain activity during this behavior is measured in order to unravel the neurobiological underpinnings of this behavior. Thus, in these procedures behavioral tests described under 3.4.4.1 will be combined with a "neuro-measurement" technique. The techniques to measure brain activity in awake rodents in our lab are:
Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity (virus injections prior to testing necessary to visualize calcium release)
Measure-5) functional magnetic resonance imaging (fMRI) to assess whole-brain activity

The general organization is:
A) to establish and validate the measurement techniques in animal *models* for compulsive behavior – a paradigm is selected (in 3.4.4.1).
B) to measure the neuronal activity parameter in the animal *models* –will deliver data of neuronal activity during compulsive behavior.
C) to establish and validate the measurement techniques when *components* of compulsive behavior are studied in animals showing compulsive behavior – in pilot experiments a satisfactory paradigm is selected.
D) to measure the neuronal activity parameter in the animal models while they are engaged in one of the *components* –will deliver data of neuronal activity during e.g. habit formation in compulsive animals.

Depending on the results with 3.4.4.1 we will start in A) with three models (e.g. a transgenic mouse, a rat pharmacological and a rat addiction model) and three measurement techniques (electrophysiology, fast-scan cyclic voltammetry and fMRI). Electrophysiology and voltammetry will be used in all three models, but fMRI will be restricted to the rat models. We will then perform formal measurements under B). We will start in C) adding the component that in 3.4.4.1 turned out to be the most interesting one – formal measurements are then carried out under D).

All of the above measuring techniques (3.4.4.2) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement. Measure-1 through Measure-4 require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays. Measure-5 requires head re-straining because movement artefacts will otherwise prevent measurements. The use of behavioral tests in combination with Measure-5 is restricted and will mainly concern inducing a compulsive phenotype before measurements with fMRI.

3.4.4.3 Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation

To discover causal relationships between brain activity and behavior, behavioral tests described under 3.4.4.1 will be combined with a "neuro-intervention" technique, which will often involves invasive procedures. The techniques to interfere with brain activity in awake rodents in our lab are:
Intervent-1) deep-brain stimulation (DBS; high-frequency electric stimulation of brain tissue via intra-cranially implanted micro-electrodes)
Intervent-2) pharmacogenetics (virus injections prior to testing necessary for expression of drug-sensitive receptors in specific cell populations and subsequent intervention by drug application)
Intervent-3) optogenetics (virus injections prior to testing necessary for expression of light-sensitive receptors in specific cell populations and subsequent intervention by light application)
Intervent-4) brain lesions (both permanent and transient inactivation of specific brain regions via intracranial injection of substances)
Intervent-5) pharmacological treatments (both systemic and intracranial application of neuro-active drugs)

The general organization is:

A) to establish and validate the intervention techniques in animal *models* for compulsive behavior –a satisfactory paradigm is selected (in 3.4.4.1).
B) to interfere with the neuronal activity in the animal *models* –will deliver behavioral data of intervention-induced alterations in compulsive behavior.
C) to establish and validate the intervention techniques when *components* of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory paradigm is obtained; that paradigm will be validated.
D) to interfere with the neuronal activity in the animal models while they are engaged in one of the *components* –will deliver behavioral data of intervention-induced alterations in habit formation etc in compulsive animals.

Depending on the results with 3.4.4.1 and 2 we will start in A) with 3 models (e.g. a transgenic mouse, a rat pharmacological and a rat addiction model) and 3 intervention techniques (DBS, optogenetics, pharmacogenetics). We will then perform formal experiments under B).
We will start in C) with the component that gave the most promising results in the previous studies and combine that with the three models.

Most of the above techniques (3.4.4.3) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement and tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays.

3.4.4.4 Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation
Based on the results and/or the experience with behavioral and neurophysiological/neurochemical measurements, and with neural interventions, we will use combinations of one of the neuro-intervention with one of the neuro-measurement techniques described under 3.4.4.2 and 3.4.4.3 in ongoing behavior, as described under 3.4.4.1. Such combinations will allow the assessment of whether manipulated brain activity takes place, and whether it manifests time-locked to the ongoing behavior. When the results obtained in the measurement and/or intervention section that combined experiments are indicated, the general organization is:
A. to establish and validate the combination of intervention and measurement techniques in animal models for compulsive behavior, either with or without engagement in one of the components – in pilot experiments a satisfactory paradigm is selected for further studies.
B. to interfere with the neuronal activity in the animal models and measure the effect on neuronal activity – will deliver causal relations between intervention-induced alterations in behavior and neuronal activity.

3.4.4.5 Identification and characterization of neuroanatomical connections and their regulation
In a parallel approach, the interplay of neurobiological variables (e.g., neurotransmitters) and neuroanatomical pathways are investigated in the anesthetized animal, where, in the absence of movement, brain activity is easier to assess and brain stimulation is applied easier (no long-term implantation necessary). Questions that can be answered with this approach include: How do the basal ganglia propagate information (neuronal activity) between different functional subunits? Or how reactive are different subregions to neuronal input from the cortex or the midbrain (inputs activated with a stimulation technique (e.g., optogenetics and output measured with a measuring technique (e.g., voltammetry). Thus, in anesthetized animals (short-term experiments → no survival surgery), we will combine the use of one of each of the under 3.4.4.2 and 3.4.4.3 described neuro-intervention and neuro-measurement techniques. However, in the case of optogenetic interventions, this would have to be preceded by stereotactic micro-infusion of a virus to locally express light-sensitive or other proteins.

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

The coherence between the above outlined components stems from the overarching goal to identify and characterize neural substrates for compulsive behavior and its "building blocks" (i.e., provide a better understanding of the neurobiology of compulsive behavior; **see flow chart**). Specifically, in each component either behaviors or measures of brain activity that are associated with compulsivity are studied. In order to do so, a variety of motivated behaviors thought to constitute or contribute (or both) to compulsivity will be studied while neural recordings and stimulations are performed. All components

taken together are capable of accomplishing this goal. Thus, the primary logical structure of the approach is 1) to establish a specific rodent behavior of interest, then 2) to identify and measure brain regions that may be involved, then 3) to manipulate that brain region and assess its effect on behavior, and finally either 4) to assess its effect on other brain variables *during* behavior, or 5) in anesthetized animals. Although all these components are intended to be tied together by logic and temporal sequence, it is important to note that they can be executed independently or in different sequences – often, these experiments may not follow this logical path and different experiments will not be developed in a synchronous manner. For example, in case we already have information from our own studies or those from other labs indicating a specific brain region in a common behavioral assay, we may skip measuring from this brain region first and go straight to manipulating it. Or we use behavioral paradigms that are already established in our group and directly combine these with an intervention procedure based on pharmacogenetics. Or we may start out with component 5 to establish whether a certain assumed anatomical connection in the brain is capable of inducing molecular or physiological changes in another region of interest. Only after this functional connection is established, will a follow-up behavioral study be conducted.

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

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

Serial number	Type of animal procedure
1	**Establishing and characterizing rodent behavior that models compulsive behavior and its components**
2	**Identification of brain correlates of compulsive behavior and its components**
3	**Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation**
4	**Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation**
5	**Identification and characterization of neuroanatomical connections and their regulation**
6	
7	
8	
9	
10	

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101	
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.1	**Establishing and characterizing rodent behavior that models compulsive behavior and its component**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.1) is to answer the above questions 1 and 2:
A. to establish and validate animal models for compulsive behavior;
B. to characterize the tested population of animals (mice and rats) for individual differences;
C. to establish and validate behavioral methods to assess components of compulsive behavior, such as habit formation and cognitive flexibility;
D. to characterize the compulsivity in relation to its behavioral components.

The main outcome parameter of these procedures is behavior.
The behavioral procedures are meant to be used also in the subsequent appendices (3.4.4.2, 3.4.4.3 and 3.4.4.4). The results obtained form the basis to choose the best procedures and components to be used in combination with neuronal measurements and/or interventions. It is important to have these procedures validated and ready to use before the combination with invasive measurement and intervention methods, so that the behavioral procedures in animals with implants only will require some

fine-tuning.

Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

A. Introduce and validate models for compulsive behavior, so that they can be applied to answer our scientific questions.
We have chosen three rodent models as first tier models to start our studies. The choice was based on providing the best opportunity to discover a common neurobiological mechanism of the different compulsive phenotypes. The models are: Sapap3-mutant mice (compulsive grooming based on a genetic deletion of a postsynaptic density protein in the striatum); cocaine self-administration in rats (compulsive cocaine seeking and taking despite punishment); quinpirole-induced compulsive checking in rats (behavioral changes following repeated quinpirole administration). These are our first tier models, but they may be extended or replaced by other pharmacological, behavioral, genetic, and addiction models (via notification of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
The use of mice in addition to rats is based on the fact that transgenic mice such as the Sapap3-mutant mice are up to now the only model with spontaneous compulsive behavior, where no additional pharmacological treatment or behavioral training is required. The validation of the compulsive self-administration models (using cocaine or other drugs of abuse) depends on a test in which responding for the drug is punished. Compulsive animals will continue to respond despite the electrical foot shocks or substances that induce taste aversion (e.g., lithium chloride) that they receive.

B. Answer the scientific question how strong the individual differences are for the animal models of compulsivity that are validated under A.
Studying individual differences in behavior is an old approach that has proven its value over and over again. By studying the individual differences in compulsive behavior we aim to increase the chance that we will discover its underlying neurobiological mechanism. We already have experience with individual differences in two of our three first tier models, compulsive cocaine self administration (where only a limited number of rats will reach a level of compulsivity where they accept receiving foot-shocks when responding for cocaine) and Sapap3-mutant mice (where we find a high variability in the time spent grooming and where only intensely grooming mice respond to deep brain stimulation with a decrease in grooming).
We will follow this up by behaviorally testing animals from different strains and from lines with different genetic modifications. Initially, we will use two rat strains (our standard strain, Long Evans and another, to be selected on an expected clear difference in behavior) and two transgenic mouse strains (i.e. Sapap3-mutants and another, to be selected on an expected clear difference in behavior from our standard controls, C57Bl/6).
Another factor related to this is gender. For studies where animals are bred in our lab, we intend to use both males and females and this will provide us with the opportunity to collect important information on sex differences in compulsivity and the relation to underlying neurobiological mechanisms between sexes (see also section B. "The animals").

C. Introduction and validation of the "component" paradigms, so that they can be combined with the models for compulsive behavior and applied to answer our scientific questions.
The composition of compulsive behavior can be described as separate components: 1) persistence of performance, 2) elicitation of undesirable consequences, 3) escalation of symptoms over time, 4) aggravation by stress and anxiety, 5) exaggerated habit formation, 6) response inflexibility, and 7) loss of voluntary control. Whereas 1), 2), 3) and 7) are components that cannot be studied separately from the compulsive phenotype itself, 4), 5) and 6) may exist in the normal population and lower the threshold for compulsive behavior to develop. Our first interest is in the possible contribution of exaggerated habit formation and the relation of compulsivity with anxiety, but, dependent on the results obtained, the progress in the field and on the nature of the compulsivity models, we may later decide to focus on cognitive flexibility and the interaction with stress, as well. In the latter component we include the positive counterpart, i.e. possible alleviation of compulsivity by providing environmental enrichment.
Methods for fear learning may be established and validated when results with the other tests or new publications or hypotheses would make this test valuable to combine with models for compulsivity.
The cognitive flexibility component incorporates (through its training stages) also operant reward learning and decision-making. The habit test also based on operant reward responding, which, dependent

on the training schedules, leads to goal-directed or habit behavior. This is validated by using an outcome devaluation test, where the reward (outcome) is made less valuable for the animals, through pre-feeding or through association with sickness, induced by lithium chloride.
Anxiety is tested in an acute procedure, through exposure of the animal to an elevated-plus maze or an open field. Fear learning is tested in an associative procedure where the animal learns that a cue signals a foot-shock.
As these procedures need to be used in both rats and mice, they will be developed in validated in both rats and mice.
Interaction with stress generally will involve chronic exposure to various stressors (e.g. social defeat, restraint, forced swimming, foot-shocks, corticosterone administration). The positive counterpart of stress (environmental enrichment) involves social housing in regularly changing environments, e.g. large cages with many different components that are frequently altered, moved etc. We will use this type of enrichment to reverse or protect from compulsive behavior. Acute exposure to stress may also interact with compulsive behavior (only a single exposure) and will thus be implemented as well.

As it will need to be established what the best order of testing is for the combined experiments under D), in the procedures under C) we test the different options (see further under D)).

D. Answer what the relation is between compulsive behavior (in one of the models described under A and B) and separate components of that behavior (as described under C).
The three first tier models of compulsivity will be used while before or after the actual measurement of compulsive behavior the component behaviors are assessed.
Different orders of experimental tests may apply for different combinations. Thus, compulsivity testing in Sapap3-mutant mice involves the recording of spontaneous behavior. In the developmental course of the mice this may be repeated with regular intervals, so that the development of the compulsive grooming is followed. The anxiety test can also be repeated, so that over a long time course a possible relation between the two can be studied. In case of interaction with chronic stress or enrichment, compulsive grooming may be recorded both before and after the stress/enrichment exposure. Operant tests in the Sapap3-mutant mouse should preferably be performed at relatively young age, as responding is affected when the mice grow older and have a higher chance of developing lesions.
The training procedure for rats to reach a stage of compulsive cocaine self-administration is long. While anxiety testing can easily be combined with this, the other procedures require extended training periods as well, either for chronic exposure (stress, enrichment) or for (habit or flexibility) training. To keep experiments at a reasonable duration, we need to establish under C) in what order to apply a combination of tests/treatments. Similarly, establishment of experimental order apply to the combination of quinpirole treatment and the operant (habit/flexibility) tests.

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

First tier **models** of compulsivity.
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks. Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-

3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

Second tier **models** of compulsivity.
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

Components of compulsivity.
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired

and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)
- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the

experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, animals with catheters, intracranial virus injections, repeated quinpirole treatments, chronic stress exposure and all Sapap3-mutant mice will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology.

A small subset of these animals (up to 25% but likely much less) may be used for the terminal experiments under anesthesia (3.4.4.5), for measurement of neuronal activity following acute intervention with brain activity. A small subset of these animals that were exclusively tested for rewarded behavior may be available for use in other experiments, as well.

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

Pilot experiments: establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on pilot studies and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

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

Species used:

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

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Behavioral studies contain an average of 10 rats or mice for each experimental group and control groups. Individual differences will be tested in 20 animals. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour) and accounting for a range of second tier models of compulsivity we will use 1500 animals in this appendix, 500 mice and 1000 rats. Of these, approx 30% will be exposed to mild and 70% to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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

☒ No

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

D. Replacement, reduction, refinement

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

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.
We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in house. This will lead to a reduction of "breeding surplus".

Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4). However, a certain number (approx. up to 25%) of the animals in 3.4.4.1 will be transferred to one of the other procedures, most of them in 3.4.4.5 (terminal experiments under anesthesia).

Experiments will be executed in succession and, if needed, small explorative studies (pilots) will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in treatment-naïve animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the

maximum number of animals needed to obtain interpretable data.

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

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Plasma sampling in animals for the measurement of cocaine or corticosterone concentrations in rats or mice, will be within in recommended/allowed limits.

Procedures will only be performed by competent personnel, as mandatory.
Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless food-restricted or implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment (see also F.). Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

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

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

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

In a subset of cases, such as after implantation of optic fibers, intravenous catheters etc., animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary.

In some cases, food restriction needs to be combined with isolated housing, when socially housed animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and

treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used).

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

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

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

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.
7. Chronic stress-exposed animals with catheters for plasma sampling need to handled leading to repeated mild discomfort.
8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Other aspects that may compromise the welfare of the animals are:
    - Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anesthetic, or accidental severing of nerve fibers or blood vessels.
    - Inflammation in the tissue around implanted devices such as intravenous catheters.
    - During intravenous drug self-administration animals sometimes overdose.
10. All animals will be frequently monitored for possible side effects. Animals exhibiting an unexpected phenotype with discomfort will be sacrificed immediately.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 8 is inherent to surgical procedures.

Surgical procedures cannot be executed with 0% failure rate and very seldom increased postoperative bleeding leads to maximally moderate discomfort.
There is considerable variability within rodent populations regarding the sensitivity to anaesthetics and drugs.

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

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.

If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

J. Humane endpoints

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

☐ No > Continue with question K.

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

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

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

We will use 1500 animals in this appendix, 500 mice and 1000 rats. Of these, approx 30% will be exposed to mild and 70% to moderate discomfort.

Of the compulsivity models, Sapap3-mutants may develop lesions. We estimate that in the course of the experiments 75% will experience maximally mild discomfort and 25% max moderate. If we expect that the discomfort will further increase, the animals are euthanized.

The addiction models all undergo surgery, may experience abstinence and foot-shocks when compulsivity is validated, together leading to maximally moderate discomfort.

The optogenetic stimulation models also undergo surgery, leading to max moderate discomfort.

Quinpirole and other pharmacological models may experience transient moderate discomfort.

During fear conditioning and chronic stress, animals may also experience moderate discomfort.
All other animals (including most control groups) wil experience no more than mild discomfort (food restriction plus behavioral testing).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

Some animals (up to 25%) that were used for rewarded behavior in this appendix are available for use in other experiments and will not be sacrificed under this appendix (the majority of these 25% percent will be used for the terminal experiments under appendix 3.4.4.5).

All other animals will receive an overdose of Nembutal and perfused for brain fixation, immunohistochemistry and histology.

Subsequently, protein expression following virus injections, localization of implanted fibers and possible brain pathology in compulsivity models will be assessed.

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

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

☒ Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 Type of animal procedure: **Identification of brain correlates of compulsive behavior and its components**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.2) is to answer the above question 3:
- to measure brain activity in behavioral paradigms for compulsive behavior and its components (such as habit formation or cognitive flexibility) in order to unravel the neurobiological underpinnings of this behavior.

The main outcome of these procedures in neuronal activity, in combination with behavior.
Thus, in these procedures behavioral tests described under 3.4.4.1 will be combined with one of the following "neuro-measurement" techniques to measure brain activity in awake rodents in our lab:
Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity
Measure-5) functional magnetic resonance imaging (fMRI) to assess whole-brain activity.

A maximum of two "neuro-measurement" techniques will be used in a single animal. In the vast majority

of cases only a single measurement technique is used in a single animal.
Both neuronal activity and neurotransmitter release are studied and measurements focus on both local and global processes. We need such an array of measurement techniques to increase the chance that we can identify the neurobiological correlates of the behavior studied and thus find targets for subsequent (3.4.4.3) intervention experiments.
Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

The general organization is:
A) to establish and validate the measurement techniques in animal models for compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory paradigm is obtained; that paradigm will be used (in 3.4.4.1).
B) to measure the neuronal activity parameter in the animal models – will deliver data of neuronal activity during compulsive behavior.
C) to establish and validate the measurement techniques when components of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory paradigm is obtained; that paradigm will be used.
D) to measure the neuronal activity parameter in the animal models while they are engaged in one of the components – will deliver data of neuronal activity during habit formation etc. in compulsive animals.

All neuro-measurement techniques (3.4.2.2) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement. Measure-1 to 4 require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays. Measure-5 (and in some cases Measure-4, when needed, so also D) requires head re-straining because movement artefacts will otherwise prevent measurements. The use of behavioral tests in combination with 4) is restricted and will mainly concern inducing a compulsive phenotype before measurements of possible alterations of resting state activity with fMRI. Pilot experiments (C) to combine conditioning tests (both appetitive and aversive) with fMRI measurements will be initiated and should lead to formal experiments under D.

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

Neuro-measurements are carried out in animal models of compulsivity and their controls. We'll start with our three first tier models (Sapap3-mutant mice, quinpirole-treated rats and cocaine self-administering rats) and combine these with 3 measurement techniques (electrophysiology, fast-scan cyclic voltammetry and fMRI). Electrophysiology and voltammetry will be used in all 3 models, but fMRI will be restricted to rats in the cocaine self-administration and quinpirole models.

When such measurements are combined with components of compulsivity the standard is to test anxiety and one other component (i.e., either habit formation, cognitive flexibility, sensitivity to stress, or fear conditioning). We'll first focus on habit formation, later on cognitive flexibility.

This procedure can consist of the following steps:

1. All animals (wild type or genetically manipulated) are housed together in single-sex groups until they become at least young adults (8 weeks of age). Two weeks before the start of behavioral experiments animals will be handled and weighed frequently.
2. Measurement equipment is implanted into the animals' brains through holes that are drilled into the skull (under adequate anesthesia and analgesia). In case of calcium imaging (Measure-4), a virus that will express Ca-indicating proteins, is infused (under adequate anesthesia and analgesia; at least 3-4 weeks recovery from this surgery to allow the virus to express). Animals will recover from anesthesia for at least one week.
3. Measurements in a model for compulsive behavior (group B) or compulsive behavior *and* its components (group D). Pilot experiments in groups A and C are used to establish the optimal sequence and timing of events.

Procedures Measure-1 to 4 (electrophysiology, voltammetry, microdialysis, Ca-imaging):
After the animals are connected to the recording/measuring set-up, they can move freely in the test box or test maze. The total time in the test will vary between 2-9 h. Daily electrophysiology and voltammetry

measurements can continue for up to 3 months. Microdialysis measurements can be repeated once. Ca-imaging can be repeated. Pilot experiments will be needed to establish the frequency and maximum number of measurements – a task that will involve frequent consultation of the IvD.

Procedures Measure-4 and Measure-5 (Ca- and fMRI-imaging):
Scanning animals in a MRI scanner (and in some cases calcium imaging (when using chronically implanted imaging windows)) requires head restraining to minimize head-movement-induced artefacts in the measurements. We follow a training protocol of 5 consecutive days with a duration of up to one hour each which reduces stress responses (corticosterone levels and observed restrained behavior). Non-coping animals will be removed from the experiment. After restraint training sessions concluded, animals will be transported to the MRI scanner (or calcium imaging apparatus). There the animals will be placed inside the scanner bore in our restrainer device, which has room for a custom build head coil specifically designed for rodents (and room for connecting the calcium imaging equipment).
Training duration: 1-2 weeks. Both Ca- and fMRI imaging may be repeated. Pilot experiments over the course of several months will be needed to establish the frequency and maximum number of measurements.

Sequence of experiments. Most of the models of compulsivity and also most of the components need acquisition/treatment periods of several weeks. The optimal sequence of events (compulsivity acquisition, intracranial implantation and measurements, acquisition of components) may vary: in group B the sequence may be implantation, measurement during compulsivity acquisition and expression; in group D: compulsivity acquisition, implantation, measurement during component acquisition (habit, flexibility, fear conditioning) or, alternatively, component (chronic stress or enrichment), implantation, compulsivity acquisition. The most suitable sequence (in terms of measurement success and animal discomfort) will be selected in pilot experiments in C).

*First tier **models** of compulsivity (**text identical to 3.4.4.1 is indicated in italics**).*
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks. Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final

phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

Second tier ***models*** *of compulsivity.*
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

Components *of compulsivity.*
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily

exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)
- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, animals with catheters, intracranial virus injections, repeated quinpirole treatments, chronic stress exposure and all Sapap3-mutant mice will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology.

Some of these animals (up to 25%) may be used for the terminal experiments under anesthesia (3.4.4.5), for measurement of neuronal activity following acute intervention with brain activity. A subset of these animals that were exclusively tested for behavior may be available for use in other experiments, as well. As all animals are implanted with electrodes, equipped with head posts etc. and have been through long behavioral procedures, they are not available for re-use in any protocol involving behavior.

After completion of the collection of data, the animals will be sacrificed (overdose of Nembutal and perfused for brain fixation) and their brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes, or stains to assess the effects of electrode stimulation). In the case of electrophysiology and voltammetry a small electrolytic lesion under proper isoflurane anesthesia will precede the Nembutal treatment and perfusion.

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

Pilot experiments: Establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

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

Species used:

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

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be

determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuromeasurement studies (3.4.4.2) contain an average of 20 animals (experimental group plus controls) plus 2 extra rats or mice for each experimental group and control groups, compared to the purely behavioral experiments of 3.4.4.1. This is to account for drop-out because of mis-placement and/or technical problems over the course of the experiments. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour; on average measurements and interventions are taking place on no more than a third of the overall experimental training days) we will use 1050 animals in this appendix, 350 mice and 700 rats. All (100%) will be exposed to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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

☒ No

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

D. Replacement, reduction, refinement

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

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.
We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals. The basic testing of these intervention- and measurement-techniques will be performed as much as possible prior to performing an animal experiment.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in house, this will lead to a reduction of "breeding surplus".

Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4).

The measurement techniques that will be most frequently used (electrophysiology and fast-scan cyclic voltammetry) have been developed to allow chronic recordings in each animal. Thus, we will strive to perform experiments where each animal is his/her own control if possible (e.g. stimulation on vs stimulation off – this is also the way in which the clinical experiments are performed). In general, this also increases power and decreases the number of animals required.

Ca-imaging will be carried out using fiber implants and the animals can move around freely during recordings. The use of imaging windows which requires head fixation (and head fixation training) will be

avoided as much as possible.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in control animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the minimum number of animals needed to obtain interpretable data.

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

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment. Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

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

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

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

In a subset of cases, such as after implantation of optic fibers, intravenous catheters etc., animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary.

In some cases, food restriction needs to be combined with isolated housing, when socially housed animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used.

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

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

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

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.
7. Chronic stress-exposed animals with catheters for plasma sampling need to handled leading to repeated mild discomfort.
8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Handling animals to connect implanted electrodes etc. to measurement equipment and, following behavioral and measurement sessions, disconnect them leads to repeated mild discomfort.
10. Rats used for fMRI measurements will undergo restraint training, that will not exceed moderate discomfort. Rats showing signs of non-coping will be taken out of the experiment.
11. Other aspects that may compromise the welfare of the animals are:

- Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anaesthetic, or accidental severing of nerve fibers or blood vessels.
- Inflammation in the tissue around implanted devices such as intravenous catheters.
- During intravenous drug self-administration animals sometimes overdose.
- Damage or loss of the head-stage/connector on the skull may lead to moderate discomfort. Animals will be taken out of the experiments when this happens.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 7 is inherent to surgical procedures.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anesthetics and drugs.

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

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.
If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

Rats will be extensively handled and carefully trained for fMRI measurements. Rats that do not cope with the restraining training, will be taken out of the experiment. The restraining itself will be carried out under transient, light isoflurane anesthesia.

J. Humane endpoints

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

☐ No > Continue with question K.

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

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

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

Level of discomfort: Neuro-measurement studies (as described here in 3.4.4.2 (and the behavioral aspect in 3.4.4.1)) last up to 3 months; up to 6 months when two are combined. In the majority of

paradigms, we food-deprive the animals (mild discomfort). Exceptions are drug self-adminstration studies (also mild discomfort due to drug withdrawal and catheter implantation) and studies only looking at measures of anxiety (mild discomfort due to experiencing fear and anxiety; or pain due to foot shocks) and spontaneous behavior (no discomfort (if not implanted with a headcap)). Of the SAPAP3 mutant mice, up to 50% will experience mild discomfort due to small skin lesions inflicted by excessive grooming (phenotype); the other 50% will be used before this phenotype develops. In addition, most animals will receive head implants or intracranial injections during a stereotaxic surgery for the measurement of brain activity. The recovery of this surgery is deemed moderate discomfort (for one week). Following recovery, wearing a cement headcap and being tethered to a commutator frequently will induce mild discomfort. Thus, we estimate 100% of the animals to experience mild discomfort throughout the experiments, with a period of moderate discomfort for up to one week after stereoxic surgeries. A small percentage of rats (up to 10% will undergo head restraining several times, which induces moderate discomfort.

In total, we estimate that of the 350 mice, 350 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereotactic surgeries or during training of head restraining.

Of the 700 rats, 700 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereotactic surgeries or during head restraining

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

All animals will receive an overdose of Nembutal and perfused for brain fixation, immunohistochemistry and histology.

Subsequently, protein expression following virus injections, localization of implanted fibers and possible brain pathology in compulsivity models will be assessed.

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

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

☒ Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101	
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.3	**Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.3) is to answer the above question 4 and 5:
- to manipulate brain activity in behavioral paradigms for compulsive behavior and its components.

The main outcome of these procedures is behavior.
Once a behavioral strategy is established and the desired behavior is detected, brain activity during this behavior is manipulated in order to unravel the neurobiological underpinnings of this behavior. To discover causal relationships between brain activity and behavior, behavior will be measured while brain activity is manipulated using the following interventions:

The aim of these procedures is
Intervent-1) Deep-brain stimulation (DBS)
Intervent-2) pharmacogenetics
Intervent-3) optogenetics

Intervent-4) lesions
Intervent-5) pharmacological treatments

A maximum of two "neuro-intervention" techniques will be used in a single animal. In the vast majority of cases only a single intervention technique is used in a single animal.

These techniques allow interventions of both local and global processes, with different levels of spatial, cellular and pharmacological selectivity. We need such an array of intervention techniques to increase the chance that we can identify the neurobiological underpinnings of the behavior studied and find ways to alter compulsive behavior.
Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

The general organization is:
A) to establish and validate the intervention techniques in animal models for compulsive behavior – in pilot experiments a satisfactory paradigm is selected for further use (in 3.4.4.1);.
B) to alter the compulsive behavior in the animal models –will deliver data of efficacy potential of target structures and/or cellular processes.
C) to establish and validate the intervention techniques when components of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments a satisfactory paradigm is selected for further studies.
D) to alter behavior in the animal models while they are engaged in one of the components – will deliver efficacy potential data of target structures and/or cellular processes.

All neuro-intervention techniques will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement (Intervent-1,-2,-3, and -5). Exceptions are Intervent-4, where a one-off local microinjection is performed. In Intervent-2 and Intervent-5 pharmacological agents can be administered peripherally or centrally through the implanted cannula.
Intervent-1 and Intervent-3 involve continuous application of electrical or light pulses and therefore require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays.

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

Neuro-interventions are carried out in animal models of compulsivity and their controls. We'll start with our three first tier models (Sapap3-mutant mice, quinpirole-treated rats and cocaine self-administering rats) and combine these with 3 intervention techniques (deep brain stimulation, optogenetics, pharmacogenetics).

This procedure can consist of the following steps (including steps described in 3.4.4.1, see below):
1. Animals are housed together until they become at least young adults (6-8 weeks of age). Then they are handled and weighed every week.
2. In case of pharmacogenetics (Intervent-2) and optogenetics (Intervent-3), viruses that will express proteins that will make infected neurons sensitive to pharmacological or optical treatment, are infused intracranially. If possible, in the same surgical session, intervention devices are implanted into the animals' brains through holes that are drilled into the skull (under adequate anaesthesia and analgesia). Depending on the technique, the equipment consists of electrodes (Intervent-1), a guide cannula to enable the infusion of pharmacological agents (Intervent-2,-4, and -5), or fiber optics (Intervent-3).
3. Animals will acutely recover from anaesthesia in their home cages on a heating plate. Subsequently, long-term recovery from surgery will last one week, in which the animals will not undergo any additional experimental procedures causing discomfort.
4. Training in a model for compulsive behavior and/or its components.
5. Application of "Neuro-intervention" techniques to alter behavior in awake rodents behaving in paradigms listed below.

Procedures Intervent-1 and -3:
After transport from the housing room to the experimental room, animals will be habituated to the room

for one hour. Then animals will be introduced to the testing environment and connected to the intervention set-up. In some cases, the head-implanted equipment (if present) is connected to the recording set-up by a cable that runs through an optical (C) or electrical (A) commutator (swivel) mounted above them, allowing free movement in the experimental cage. In other cases, a commutator is not required (D and E) or a wireless device is used (A). After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be disconnected, removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for connecting and disconnecting the animals]. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to maximally 3 months in some cases]
At the end of the experiment, the animals will be given an overdose of Nembutal and perfused for brain fixation, histology, and immunohistochemistry. In case of DBS (A), a weak current is applied prior to perfusion to the stimulating electrode for up to 30 seconds to mark the position of the electrodes with a small electrolytic lesion under proper isoflurane anaesthesia (no discomfort for the animal).

Procedures Intervent-2 and -5:
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. On some days, animals will be infused intracranially with pharmacological agents by introducing an infusion cannula into the previously implanted guide cannula. Subsequently, the animals are introduced to the testing environment. After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for infusing the animals and letting the intervention drug act]. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to maximally 3 months in some cases]
At the end of the experiment, the animals will be given an overdose of Nembutal and perfused for brain fixation, histology, and immunohistochemistry.

Procedure Intervent-4:
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. Subsequently, the animals are introduced to the testing environment. After behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately test 1-6 hours. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to maximally 3 months in some cases]
At the end of the experiment, the animals will be given an overdose of Nembutal and perfused for brain fixation, histology, and immunohistochemistry.

Sequence of experiments. Most of the models of compulsivity and also most of the components need acquisition/treatment periods of several weeks. The optimal sequence of events may vary: in some cases the sequence may be implantation, measurement during compulsivity acquisition and expression; in other cases the sequence compulsivity acquisition, implantation, measurement during component acquisition (habit, flexibility, fear conditioning) might be preferred or, alternatively, component (chronic stress or enrichment), implantation, compulsivity acquisition. The most suitable sequence (in terms of measurement success and animal discomfort) will be selected in pilot experiments in C).

*First tier **models** of compulsivity (**text identical to 3.4.4.1 is indicated in italics**).*
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks.

Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

*Second tier **models** of compulsivity.*
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

***Components** of compulsivity.*
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a

Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)

- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, all animals will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology. The brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes or cannulae, or stains to assess the effects of electrode stimulation). In the case of virus injections the expression of the light- or drug-sensitive proteins will be visualized.

As all animals are implanted with electrodes, equipped with head posts etc. and have been through long behavioral procedures, they are not available for re-use in any protocol involving behavior.

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

Pilot experiments: Establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant – all of which will be communicated to and checked by the IvD.

B. The animals

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

Species used:

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

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-

house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuro-intervention studies (3.4.4.3) contain an average of 20 animals (experimental group plus controls) plus 2 extra rats or mice for each experimental group and control groups, compared to the purely behavioral experiments of 3.4.4.1. This is to account for drop-out because of mis-placement and/or technical problems over the course of the experiments. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour; on average measurements and interventions are taking place on no more than a third of the overall experimental training days) we will use approximately 1050 animals in this appendix, 350 mice and 700 rats. All (100%) will be exposed to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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

☒ No

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

D. Replacement, reduction, refinement

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

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.
We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals. The basic testing of these intervention- and measurement-techniques will be performed as much as possible prior to performing an animal experiment.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.
We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in

house, this will lead to a reduction of "breeding surplus".

Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4).

The intervention techniques that will be most frequently used (deep brain stimulation and optogenetics) allow repeated interventions and interventions using a wide range of different parameters in each animal. Thus, we will strive to perform experiments where each animal is his/her own control if possible (e.g. stimulation on vs stimulation off – stimulation A vs stimulation B etc; this is also the way in which the clinical experiments are performed). In general, this also increases power and decreases the number of animals required.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in control animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the maximum number of animals needed to obtain interpretable data.

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

All surgical procedures resulting in animal suffering or pain will be performed under adequate anaesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment. Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

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

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

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

In most cases, such as after implantation of optic fibers, intravenous catheters etc., animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary. In some cases, food restriction needs to be combined with isolated housing, when socially housed

animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used).

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

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

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

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, Increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.

7. Chronic stress-exposed animals with catheters for plasma sampling need to be handled leading to repeated mild discomfort.
8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Handling animals to connect implanted electrodes etc to measurement equipment and, following behavioral and measurement sessions, disconnect them leads to repeated mild discomfort.
10. Neuro-intervention is used to induce behavioral changes. While the aim is to reduce compulsive (pathological) behavior, it cannot be completely avoided that we may also sometimes induce unwanted behavior. In the pilot experiments we will carefully select the parameters for DBS and optogenetic stimulation, the targets for lesions, local microinfusions of pharmacological agents so that no extra discomfort is caused when the formal experiments are performed.
11. Other aspects that may compromise the welfare of the animals are:
    - Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anaesthetic, or accidental severing of nerve fibers or blood vessels.
    - Inflammation in the tissue around implanted devices such as intravenous catheters.
    - During intravenous drug self-administration animals sometimes overdose.

Damage or loss of the head-stage/connector on the skull may lead to moderate discomfort. Animals will be taken out of the experiments when this happens.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 and 9 and 10 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 8 is inherent to surgical procedures.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and very seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anaesthetics and drugs.

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

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.

If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

J. Humane endpoints

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

☐ No > Continue with question K.

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

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older

(> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

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

Level of discomfort: Neuro-intervention studies (as described here in 3.4.4.3 (and the behavioral aspect in 3.4.4.1)) last up to 3 months; up to 6 months when two are combined. In the majority of paradigms, we food-deprive the animals (mild discomfort). Exceptions are drug self-adminstration studies (also mild discomfort due to drug withdrawal and catheter implantation) and studies only looking at measures of anxiety (mild discomfort due to experiencing fear and anxiety; or pain due to foot shocks) and spontaneous behavior (no discomfort (if not implanted with a headcap)). Of the SAPAP3 mutant mice, up to 50% will experience mild discomfort due to small skin lesions inflicted by excessive grooming (phenotype); the other 50% will be used before this phenotype develops. In addition, most animals will receive head implants or intracranial injections during a stereotaxic surgery for the measurement of brain activity. The recovery of this surgery is deemed moderate discomfort (for one week). Following recovery, wearing a cement headcap and being tethered to a commutator frequently will induce mild discomfort. Thus, we estimate 100% of the animals to experience mild discomfort throughout the experiments, with a period of moderate discomfort for up to one week after stereoxic surgeries. A small percentage of rats (up to 10% will undergo head restraining several times, which induces moderate discomfort.

In total, we estimate that of the 350 mice, 350 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereotactic surgeries or during training of head restraining (→ cumulative moderate).

Of the 700 rats, 700 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after steretactic surgeries or during head restraining (→ cumulative moderate).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

All animals will receive an overdose of Nembutal and perfused for brain fixation, immunohistochemistry and histology.

Subsequently, protein expression following virus injections, localization of implanted fibers and possible brain pathology in compulsivity models will be assessed.

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

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

☒ Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101	
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		3.4.4.4	**Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.4) is to answer the above questions 3, 4, and 5:
- to identify neuroanatomical connections between brain regions involved in compulsive behavior (and its components) and to characterize how these brain regions interact with and regulate each other.

Neuro-intervention and neuro-measurement in awake behaving animals (only reached in a relatively small number of animals): Brain activity will be manipulated (excitation or inhibition) at the neuronal or network level using pharmacology, optogenetics, pharmacogenetics, deep-brain stimulation (DBS) or by performing lesions (see 3.4.4.2). These techniques can facilitate or disrupt the activity of a group of neurons in a local region (e.g., optogenetics), neurotransmitter systems or entire brain networks (e.g., DBS). Such interventions will allow us to establish causal relationships between behavior and neural correlates of interest, which is one of the key aims of this proposal. For these experiments, we will measure the difference in neuronal responses and behavior (simultaneously) between a baseline

time when the manipulation had not been performed and following this intervention. Measurements will be collected using neurobiological activity using calcium imaging, electrophysiology, electrochemistry, microdialysis, and fMRI (also see 3.4.4.3).

The main outcome parameter of these procedures is neuronal activity, in combination with behavior.
Thus, in these procedures "neuro-measurement" and "neuro-intervention" techniques described under 3.4.4.2 and 3.4.4.3, respectively, will be combined to study brain activity and interaction between brain systems in the awake behaving animal. Thus, in these procedures behavioral tests described under 3.4.4.1 will be combined with one of the following "neuro-measurement" techniques to measure brain activity in awake rodents in our lab (techniques previously described in **3.4.4.2**):

Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity
Measure-5) functional magnetic resonance imaging (fMRI) to assess whole-brain activity.

"Neuro-intervention" techniques to measure brain activity in awake rodents in our lab (techniques previously described in **3.4.4.3**) are:
Intervent-1) Deep-brain stimulation (DBS)
Intervent-2) pharmacogenetics
Intervent-3) optogenetics
Intervent-4) lesions
Intervent-5) pharmacological treatments

One "neuro-measurement" technique will be combined with one "neuro-intervention" technique.

Both neuronal activity and neurotransmitter release are studied and measurements focus on both local and global processes. We need such an array of measurement and intervention techniques to increase the chance that we can identify the neurobiological correlates of the behavior studied and thus find targets for subsequent (3.4.4.3) intervention experiments.
Below the general organization of the experiments is outlined. We've chosen a selected number of compulsivity models, component behaviors and measurement techniques that will be the first focus of our attention. The remaining (second tier) models, components and techniques will later be used to extend findings and solve questions that are still unanswered after the first tier of experiments.

The general organization is:
A) to establish and validate the combination of measurement and intervention techniques in animal models for compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory solution is obtained.
B) to measure the neuronal activity parameter in the behaving individuals of animal models for compulsive behavior in response to neuro-intervention – will deliver data of neuronal activity during compulsive behavior.
C) to establish and validate the combination of measurement and intervention techniques when components of compulsive behavior are studied in animal models of compulsive behavior – in pilot experiments the first steps are taken, until a satisfactory solution is obtained.
D) to measure and manipulate neuronal activity parameters in the animal models and controls while they are engaged in one of the components – will deliver data of neuronal activity during habit formation etc. in compulsive animals.

All neuro-measurement techniques (3.4.4.2) will require intracranial (technical) implants mounted to the skull of the animals with screws and dental cement. Techniques Measurement-1 to -4 require tethering of the animals from their cement head caps (implants differ slightly depending on the technique) to commutators (connected with technical equipment) to allow animals to move freely during the behavioral assays.
Measurement-5 (and in some cases measurement-4) requires head re-straining because movement artefacts will otherwise prevent measurements. Pilot experiments (C) to combine conditioning tests (both appetitive and aversive) with fMRI measurements will be initiated and should lead to formal experiments under D.

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

Neuro-measurements and neuro-interventions are carried out in animal models of compulsivity and their controls. We'll start with our three first tier models (Sapap3-mutant mice, quinpirole-treated rats and cocaine self-administering rats) and combine these with 3 measurement techniques (electrophysiology, fast-scan cyclic voltammetry and fMRI) and a selection of intervention techniques (see above). Electrophysiology and voltammetry will be used in all 3 models, but fMRI will be restricted to rats in the cocaine self-administration and quinpirole models.
When such measurements are combined with components of compulsivity the standard is to test anxiety and one other component (i.e., either habit formation, cognitive flexibility, sensitivity to stress, or fear conditioning). We'll first focus on habit formation, later on cognitive flexibility.
This procedure can consist of the following steps:

1. All animals (wildtype or genetically manipulated) are housed together in single-sex groups until they become at least young adults (8 weeks of age). Two weeks before the start of behavioral experiments animals will be handled and weighed frequently.
2. Measurement and intervention equipment is implanted into the animals' brains through holes that are drilled into the skull (under adequate anesthesia and analgesia). In case of calcium imaging (measurement-4), a virus that will express Ca-indicating proteins, is infused (under adequate anesthesia and analgesia; at least 3-4 weeks recovery from this surgery to allow the virus to express). Animals will recover from anesthesia for at least one week.
   Depending on the technique, the equipment consists of electrodes (Measreument-1 and measurement-2), a guide cannula to enable lowering of electrodes (measurement-2) or a semipermeable membrane (measurement-3), fiber optics (measurement-4), or a post for head fixation (measurement-4 and measurement-5). In case of calcium imaging (measurement-4), a virus that will express proteins that make calcium fluorescent and thus optically detectable, is infused.
   During the same surgery, intervention devices are implanted into the animals' brains. Depending on the technique, the equipment consists of electrodes (intervention-1), a guide cannula to enable the infusion of pharmacological agents (intervention-2, intervention-4, and intervention-5), or fiber optics (intervention-3). In case of pharmacogenetics (intervention-2) and optogenetics (intervention-3), a virus that will express proteins that will make infected neurons sensitive to pharmacological (e.g., clozapine-N-oxide) or optical (e.g., light manipulating so-called opsins) treatment, is infused.
3. Measurements and interventions in a model for compulsive behavior (group B) or compulsive behavior *and* its components (group D). Pilot experiments in groups A and C are used to establish the optimal sequence and timing of events.

The measurement and intervention procedures can consist of the following steps (including steps described in 3.4.4.1, see below):

Measurement-1 to -4 (electrophysiology, voltammetry, microdialysis, Ca-imaging):
After the animals are connected to the recording/measuring set-up, they can move freely in the test box or test maze. The total time in the test will vary between 2-9 h. Daily electrophysiology and voltammetry measurements can continue for up to 3 months. Microdialysis measurements can be repeated once. Ca-imaging can be repeated. Pilot experiments will be needed to establish the frequency and maximum number of measurements.

Measurement-4 or -5 (Ca- & fMRI-imaging):
Scanning animals in a MRI scanner (and in some cases calcium imaging) requires head restraining to minimize head-movement-induced artefacts in the measurements. We follow a training protocol of 5 consecutive days with a duration of up to one hour each which reduces stress responses (corticosterone levels and observed restrained behavior). Non-coping animals will be removed from the experiment.
After restraint training sessions concluded, animals will be transported to the MRI scanner (or calcium imaging apparatus). There the animals will be placed inside the scanner bore in our restrainer device, which has room for a custom build head coil specifically designed for rodents (and room for connecting the calcium imaging equipment).
Training duration: 1-2 weeks. Both Ca- and fMRI imaging may be repeated. Pilot experiments will be needed to establish the frequency and maximum number of measurements.

Intervention-1 or -3 (DBS & optogenetics):
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. Then animals will be introduced to the testing environment and connected to the intervention set-up. In some cases, the head-implanted equipment (if present) is connected to the

recording set-up by a cable that runs through an optical (intervention-3) or electrical (intervention-1) commutator (swivel) mounted above them, allowing free movement in the experimental cage. In other cases, a commutator is not required (intervention-4 and -5) or a wireless device is used (intervention-1). After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be disconnected, removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for connecting and disconnecting the animals].

Intervention-2 or -5 (pharmacogenetics & pharmacological treatments):
After transport from the housing room to the experimental room, animals will be habituated to the room for one hour. On some days, animals will be infused intracranially with pharmacological agents by introducing an infusion cannula into the previously implanted guide cannula. Subsequently, the animals are introduced to the testing environment. After neuro-intervention during behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately 2-7 h [test 1-6 hours + ~1 hour for infusing the animals and letting the intervention drug act].

Intervention-4 (lesions):
After transport from the housing room to the experimental room, animals (with excitotoxic lesions of specific brain regions of interest) will be habituated to the room for one hour. Subsequently, the animals are introduced to the testing environment. After behavioral testing [duration: 1-6 hours; see 3.4.4.1], the animals will be removed from the testing environment, returned to their home cages, and transported back to the housing room. In total, one session will take approximately test 1-6 hours. [frequency: up to one times daily, for the entire length of a behavioral paradigm (see 3.4.4.1) – up to 3 months]

Sequence of experiments. Most of the models of compulsivity and also most of the components need acquisition/treatment periods of several weeks. The optimal sequence of events (compulsivity acquisition, intracranial implantation and measurements, acquisition of components) may vary: in group B the sequence may be implantation, measurement during compulsivity acquisition and expression; in group D: compulsivity acquisition, implantation, measurement during component acquisition (habit, flexibility, fear conditioning) or, alternatively, component (chronic stress or enrichment), implantation, compulsivity acquisition. The most suitable sequence (in terms of measurement success and animal discomfort) will be selected in pilot experiments in C).

*First tier **models** of compulsivity (**text identical to 3.4.4.1 is indicated in italics**).*
1. Sapap3-mutant mice are tested for spontaneous compulsive grooming behavior by introduction in a relatively large open field, where they are left for approximately 30-90 min. Grooming behavior generally increases when the animals get older and testing is repeated with approximately a monthly frequency. Animals are regularly (first weekly, when bare spots of skin develop, daily) monitored. They are removed from the experiment and euthanized when discomfort exceeds moderate. Sapap3-mutants are an example of the group of genetic compulsivity models - all showing increased grooming behavior. Other genetically manipulated lines may be added to or replace the Sapap3 mice in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available.
Observation period: up to 10 months. Observation test: once monthly for 1-2 h.

2. Quinpirole-treated animals are treated with quinpirole on a daily or twice weekly basis. After the administration they are placed in an open field, T-maze or other environment that they can explore. Compulsive behavior is maximal after 10-15 injections and may remain present for one to several weeks. Compulsive behavior is tested by observation of checking the open field, or making choices for reward collection in the T-maze (for this, animals need to be food-restricted and kept at 85±5% of their free feeding weight). Quinpirole-treated rats present an example of the group of pharmacological compulsivity models – all depending on 1-3 weeks of drug administration and showing stereotyped or ritualized behaviors. Other models may be added to or replace the quinpirole-treated rats (after consultation of the IvD), in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Quinpirole administration: 2-6 weeks; testing 2-4 weeks; total 1-3 months.
An alternative version of this procedure is to combine the quinpirole administration with an operant procedure in which chronic quinpirole also increases checking behavior. Rats are kept at 85±5% of their

free feeding weight.
Operant training: 2-4 weeks; quinpirole administration with continued training: 2 weeks; testing: 2-4 weeks; total: up to a maximum of 3 months.

3. Cocaine (or other drugs of abuse) self-administration requires the placement of an intravenous catheter (under adequate anesthesia and analgesia) for delivery of the drug. Following this, they are house separately. After a recovery period of at least one week, the animals will be allowed to self-administer drugs of abuse through this catheter over a period of up to 3 months. Blood samples will subsequently be collected at different time intervals (less than 10 times during 48 hours) using the cannulas to determine the concentration of the substance and the expression of biomarkers. The final phase includes responding for cocaine when additionally a foot shock is delivered. In the course of the training, a period of abstinence is included, which will lead to mild to moderate discomfort in the case of cocaine and moderate discomfort when heroin is used. Cocaine self-administering rats present an example of the group of addiction compulsivity models, all showing escalating self-administration and progression to validated compulsive behavior. Other models (e.g. heroin self-administration) may be added to or replace the cocaine rats in case models of higher scientific relevance to our questions or with higher chances to result in more reproducible results are available. Surgery 1-2 weeks; daily training: 3 months; testing: 1 week; total: up to a maximum of 6 months.

*Second tier **models** of compulsivity.*
4. Repeated optogenetic stimulation of the brain (e.g., medial orbitofrontal cortex) has been described in mice, but would also be applicable in rats. This involves stereotactic microinfusion of AAV in the medial orbitofrontal cortex to express light-sensitive proteins and placement of an optic fiber in the same area or in the medial striatum (under adequate anesthesia and analgesia). After a recovery period of at least three weeks, the animal is once daily stimulated while in an open field. Repeated stimulation leads to increased grooming, which is recorded 1 h after the stimulation. After withholding stimulation, grooming is increased for another two weeks.
Surgery and virus expression: 3-4 weeks; daily stimulation and testing: 1-2 weeks; further testing 1-2 weeks; total: up to a maximum of 3 months.

5. Schedule-induced polydipsia is induced when rats are trained in an operant box (maintained at 85±5% of their free feeding weight) under a reinforcement schedule, where pellets are delivered into the experimental apparatus approximately every minute. Due to this frequent, spaced out delivery of small amounts of food, a proportion of the animals strongly increase their water intake (a water bottle is present in the experimental apparatus)
Daily training & testing: up to a maximum of 3 months.

6. Signal attenuation is tested when rats or mice (maintained at 85±5% of their free feeding weight) first learn to associate reward delivery with a cue (signal) and are then exposed to the signal in the absence of reward delivery. In the final test, this group shows more irrelevant responses than a regular extinction group. Daily training 1-4 weeks; testing 1 week; total: up to a maximum of 3 months.

In the majority of cases, only a single model of compulsivity (see 1.-6. above) will be used in a single animal. In a minority of cases, a maximum of two of the six models listed above will be used in a single animal (e.g., optogenetic generation of compulsivity in SAPAP3 mice).

***Components** of compulsivity.*
1. Anxiety testing. In behavioral tests for anxiety, the animals' general anxiety is tested by measuring their avoidance of the center of an open-field box or the amount of time spent away from exposed parts of an elevated plus maze. This is a short, acute test which may be repeated e.g. throughout the life of a Sapap3-mutant, or before and after development of cocaine- or quinpirole-related compulsive behavior. No training. Test < 1 day, repeated 2-3x over a maximum of 2-6 months.

2. Habit formation. Food restricted animals (85±5% of their free feeding weight) are trained in rewarded operant tasks favoring either habitual or goal-directed behavior and tested following pre-exposure to the rewards or by induction of taste aversion by pairing the reward with e.g. lithium chloride. Alternatively, habitual or goal-directed avoidance behavior (responding to avoid a mild foot-shock) may be acquired and tested by pre-exposure to punishments (e.g., mild shock). Daily training: 1-3 months; test up to 8 days.

3. Cognitive flexibility. Food-restricted animals (85±5% of their free feeding weight) are trained to make choices in operant tasks (in operant boxes or on cross- or T-mazes) and are exposed to a novel situation during the test. Depending on the level of flexibility tested, daily training continues for 2 weeks to 3 months and flexibility can be tested in one day at several stages during acquisition. Signal attenuation holds an intermediate position between models for compulsivity and a component of compulsivity and may be applied as a flexibility test in models of compulsivity as well.

4.a. Repeated stress exposure. Animals undergo repeated/chronic stress (e.g. social defeat, restraint, forced swimming, corticosterone administration) or repeated injections of stress hormones. Daily exposure to one of the stressors. Total: 2-4 weeks
To assess the effect of stress exposure and corticosterone administration, plasma samples will be taken in some animals after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.b. Chronic environmental enrichment. Animals undergo repeated/chronic exposure to positive stimuli by continuously altering environmental enrichment of the home cage. Exposure is continuous, with daily environmental alterations. Total: 1 month
To assess the effect of enrichment, plasma samples will be taken after implantation of permanent cannulas into the jugular vein of adult animals (under adequate anesthesia and analgesia). Subsequently, animals will be housed individually.

4.c. Acute stress exposure. Animals will be exposed to restraint, foot-shocks, TMT-odor (fox urine), or social defeat. Exposure depending on the stressor type maximally 1,5 h, once, immediately before a compulsivity or other test.

5. Fear conditioning. Animals are exposed to foot shocks paired with environmental cues. Punishments include mild electrical foot shocks (delivered in an automated behavioral testing system (operant box)). Outcome measures are for example cue-induced freezing. Daily training: up to 1 week; test: 1-2 days. Potentially repeated 2 times over a maximum of 2-6 months.

In the majority of cases, models (above, 1. through 6.) and components (above, 1. through 5.) will be tested for 3 months at the maximum. However, on average tests will be substantially shorter. On the other hand, in a few cases the maximum 3 months will be exceeded: Up to three behavioral tests will be combined in such cases (3 x 3 months or 3 + 6 months = 9 months). Absolute maximum duration of such test combinations is thus 9 months.

The duration of all procedures described in appendices 3.4.4.2, 3.4.4.3, and 3.4.4.4 are fully determined by what is outlined in 3.4.4.1, with the addition that measurements and interventions are conducted in this time period.

A lot of the components need to be tested in combination with different compulsivity models in order to identify which components are most influential. However, there are a number of combinations and experimental scenarios that are not going to be employed by us, because they are not useful in targeting the questions that we are trying to investigate. In general, compulsivity models will be used in combination with a maximum of three compulsivity component tests. In no case/scenario will the cumulative discomfort exceed moderate levels (i.e., component testing will always be temporally separated).
Not going to be used:
- fMRI scanning of mice (SAPAP3 or any other mice)
- fear conditioning (component 5) and stress exposure/environmental enrichment (component 4 (a,b,c))
- fear conditioning (component 5) and quinpirole (model 2)
- fear conditioning (component 5) and optogenetic-induced compulsivity (model 2)
- signal attenuation (model 6) and optogenetic-induced compulsivity (model 2)
- stress exposure/environmental enrichment (component 4 (a,b,c)) and optogenetic-induced compulsivity (model 2)

Testing of females. When we use female animals, estrous cycle will be checked frequently to control for potential sex hormonal effects on behavior and to determine when to conduct crucial parts of the experiments. A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

At the end of the experiment, animals with catheters, intracranial virus injections, repeated quinpirole treatments, chronic stress exposure and all Sapap3-mutant mice will be given an overdose of Nembutal and perfused for brain fixation, immunohistochemistry, and histology.

After completion of the collection of data, the animals will be sacrificed (overdose of Nembutal and perfused for brain fixation) and their brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes, or stains to assess the effects of electrode stimulation). In the case of electrophysiology and voltammetry a small electrolytic lesion under proper isoflurane anesthesia will precede the Nembutal treatment and perfusion.

As all animals are implanted with electrodes, equipped with head posts etc. and have been through long behavioral procedures, they are not available for re-use in any protocol involving behavior.

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

Pilot experiments: Establishing new or adapted behavioral procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

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

Species used:

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

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition, measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and

females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuro-intervention/measurement studies (3.4.4.4) contain an average of 20 animals (experimental group plus controls) plus 2 extra rats or mice for each experimental group and control groups, compared to the purely behavioral experiments of 3.4.4.1. This is to account for drop-out because of mis-placement and/or technical problems over the course of the experiments. Based on the present plans (most experiments will last about one month; 14 operant boxes for behavioral testing will be available for parallel use; behavioral test sessions last for about one hour; on average measurements and interventions are taking place on no more than a third of the overall experimental training days) we will use 300 animals in this appendix, 150 mice and 150 rats. All (100%) will be exposed to moderate discomfort.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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

☒ No

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

D. Replacement, reduction, refinement

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

Behavior is the important parameter measured in these experiments and the use of intact, awake animals to perform behavioral experiments is inevitable. Behavior is a complex phenomenon and the development of compulsive behavior cannot be modeled in cell cultures or lower animal species than mammals. For measurements of brain activity or for altering that activity during compulsive behavior an intact brain is needed, as well.

We have direct and intensive contact with psychiatrists who study compulsive behavior in patients and use the most advanced techniques to measure brain activity in humans. A continuous interaction with the clinicians ensures that we will always be informed of possible alternatives for animal research. However, the possibilities for invasive measurements in the human brain are restricted and the highly selective and sensitive techniques that we have available for measurement and stimulation of brain activity can as yet only be applied in (transgenic) animals. The basic testing of these intervention- and measurement-techniques will be performed as much as possible prior to performing an animal experiment.

The procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

We will use both male and female rats and mice in the case of the (transgenic) animals that are bred in house, this will lead to a reduction of "breeding surplus".
Although most of our experiments critically require behavioral naive animals, we will transfer animals to 3.4.4.5 (for further non-behavioral experimentation) whenever possible. This is not possible with animals that have intracranial implants (all of the animals in 3.4.4.2/3/4).

The measurement techniques that will be most frequently used (electrophysiology and fast-scan cyclic

voltammetry) have been developed to allow chronic recordings in each animal. Thus, we will strive to perform experiments where each animal is his/her own control if possible (e.g. stimulation on vs stimulation off – this is also the way in which the clinical experiments are performed). In general, this also increases power and decreases the number of animals required.

Ca-imaging will be carried out using fiber implants and the use of imaging windows requiring head fixation (and head fixation training) will be avoided as much as possible.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. All novel behavioral paradigms and measurement and intervention technique will first be introduced in control animals in small, pilot groups and only be used in full experiments when the procedure is validated. On basis of this previous work and experience, statistical analysis can be performed to determine the maximum number of animals needed to obtain interpretable data.

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

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia and analgesia. Close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Mice will be handled using the tube method (Hurst & West, 2010) if possible, this reduces stress resulting from interactions with the experimenter.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed if possible (unless implanted with a device, in that case animas are single-housed because they would damage each other's implants) and provided with environmental enrichment. Furthermore, animals will be handled starting up to 2 weeks before start of the experiments and they will be habituated to the experimental setup several times before testing.

Repetition and duplication

E. Repetition

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

The proposed experiments are fundamental research, an are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☐ No

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

In all cases animals will be housed solitary. This is done because otherwise cage mates will damage these implants. In such solitary housing, although animals will be physically separated, they will be able to see, smell, and hear other animals in the stable. We will limit the single housing in the duration to the minimum period necessary.
In some cases, food restriction needs to be combined with isolated housing, when socially housed animals do not receive the amounts of the food needed to maintain their body weight at 85 ± 5% of their free feeding weight. The re-introduction of animals to established groups will be carefully monitored to avoid problems of incompatibility and disrupted social relationships.

Check the answer given in procedure 3.4.4.1.

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or simulate chronic stress). All other procedures (67%) do either not produce pain or pain when is experienced, analgesia is provided (e.g., in surgical interventions adequate analgesia will be used).

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

Proper anesthesia and analgesia is used for all procedures that are not related to experimental testing (see above under "No"), which is primarily surgery.

I. Other aspects compromising the welfare of the animals

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

1. Sapap3-mutant mice show increased grooming, which by itself brings no additional discomfort, but may lead to bare spots of skin and finally to skin lesions, and maximally moderate discomfort.
2. Quinpirole injections leads to a certain period (up to 1 h) of disturbed behavior and sometimes signs of increased anxiety, associated with maximally moderate discomfort.
3. It is difficult to estimate if animals experience discomfort when they develop compulsive behavior. We estimate that by itself, increased grooming or increased operant responding does not lead to discomfort.
4. Animals addicted to cocaine or heroin do not seem to experience discomfort as long as they are able to obtain the drug. During extinction tests, animals will experience discomfort because of withdrawal symptoms. The severity varies for different drugs: cocaine abstinence is estimated as causing mild to moderate discomfort, heroin abstinence as moderate discomfort. Discomfort is highest on the first day and becomes less on subsequent days.
5. In a subset of animals (up to 33%) foot shocks will be applied. It is necessary that the animals experience these shocks in order for the behavioral tests to succeed (i.e., identify levels of compulsivity, fear/anxiety, or induce chronic stress). Animals tested for levels of compulsivity or fear conditioning will experience repeated foot-shocks in daily sessions for 1-2 weeks, leading to no more than moderate discomfort. Animals tested for the effects of stress-induced aggravation of compulsivity will experience increased stress from daily exposure to one of several stressors for the duration (2-4 weeks) of the exposure, leading to moderate discomfort.
6. Food restriction to 85 ± 5% of free feeding weight leads to initial mild discomfort, which decreases or disappears upon habituation during further training and testing.
7. Chronic stress-exposed animals with catheters for plasma sampling need to be handled leading to repeated mild discomfort.

8. Recovery from stereotactic surgery and implantation of catheters may lead to maximally moderate discomfort.
9. Handling animals to connect implanted electrodes etc. to measurement equipment and, following behavioral and measurement sessions, disconnect them leads to repeated mild discomfort.
10. Rats used for fMRI measurements will undergo restraint training, that will not exceed moderate discomfort. Rats showing signs of non-coping will be taken out of the experiment.
11. Other aspects that may compromise the welfare of the animals are:
    - Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anesthetic, or accidental severing of nerve fibers or blood vessels.
    - Inflammation in the tissue around implanted devices such as intravenous catheters.
    - During intravenous drug self-administration animals sometimes overdose.

Damage or loss of the head-stage/connector on the skull may lead to moderate discomfort. Animals will be taken out of the experiments when this happens.

Explain why these effects may emerge.

Mild to moderate discomfort in the above examples 1-7 are inherent to the models of compulsivity and to the measurement or intervention techniques, while example 7 is inherent to surgical procedures.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anesthetics and drugs.

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

Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD or veterinary officer. Possible treatment will be initiated (topically or systemically applied medication).

For intravenous drug self-administration a maximum number of drug infusions is programmed into the software controlling the infusion pump.

The intensity of foot-shocks is limited to the lowest effective combination of current strength and duration. Foot-shock intensity will never exceed 1 mA.

If animals are on a food-restriction regimen, they are weighed each day and the amount of food given is adapted to keep the weight at 85 ± 5% of free feeding weight.

Rats will be extensively handled and carefully trained for fMRI measurements. Rats that do not cope with the restraining training, will be taken out of the experiment. The restraining itself will be carried out under transient, light isoflurane anesthesia.

J. Humane endpoints

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

☐ No > Continue with question K.

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

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

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

Level of discomfort: Neuro-intervention/measurement studies (as described here in 3.4.4.4 (and the behavioral aspect in 3.4.4.1)) last up to 3 months; up to 6 months when two are combined. In the majority of paradigms, we food-restrict the animals (mild discomfort). Exceptions are drug self-adminstration studies (also mild discomfort due to drug withdrawal and catheter implantation) and studies only looking at measures of anxiety (mild discomfort due to experiencing fear and anxiety; or pain due to foot shocks) and spontaneous behavior (no discomfort (if not implanted with a headcap)). Of the SAPAP3 mutant mice, up to 50% will experience mild discomfort due to small skin lesions inflicted by excessive grooming (phenotype); the other 50% will be used before this phenotype develops. In addition, most animals will receive head implants or intracranial injections during a stereotaxic surgery for the measurement of brain activity. The recovery of this surgery is deemed moderate discomfort (for one week). Following recovery, wearing a cement headcap and being tethered to a commutator frequently will induce mild discomfort. Thus, we estimate 100% of the animals to experience mild discomfort throughout the experiments, with a period of moderate discomfort for up to one week after stereoxic surgeries. A small percentage of rats (up to 10% will undergo head restraining several times, which induces moderate discomfort.

In total, we estimate that of the 150 mice, 150 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereoxic surgeries or during head restraining.

Of the 150 rats, 150 will experience mild discomfort throughout the experiments and all of them will undergo a period of moderate discomfort for up to one week after stereoxic surgeries or during head restraining.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

Rats and mice will be killed for histological and immunohistochemical analyses.

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

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

☒ Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80101	
1.2	Provide the name of the licenced establishment.	Nederlands Herseninstituut - KNAW	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 3.4.4.5	Type of animal procedure **Identification and characterization of neuroanatomical connections and their regulation**

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The general research questions addressed in our project are:
1. How does compulsive behavior develop and is there a single or multiple form(s) of compulsivity?
2. What is the relation between compulsive behavior and its separate behavioral components?
3. How are compulsive behavior and its behavioral components encoded in the brain?
4. Which brain pathways are promising targets for therapeutic interventions such as brain stimulation?
5. What are the brain mechanisms of deep-brain stimulation (DBS) and what are the neuroanatomical connections of brain regions involved in compulsive behavior and its components?

The aim of the procedures described in this appendix (3.4.4.5) is to answer the above questions 3 and 5:
- to identify neuroanatomical connections between brain regions involved in compulsive behavior (and its components) and to characterize how these brain regions interact with and regulate each other.

The main outcome parameter of these procedures is neuronal activity (in the anesthetized animals and in brain slices).
Thus, in these procedures "neuro-measurement" and "neuro-intervention" techniques described under 3.4.4.2 and 3.4.4.3, respectively, will be combined to study brain activity and interaction between brain systems in anesthetized rodents or brain slices. Thus, this appendix is identical to 3.4.4.4 (also combines "neuro-measurement" and "neuro-intervention" techniques) except that it is carried out in an additional set of animals in the anesthetized preparation or in brain slices, so behavior is not taken into account. Furthermore, these experiments are conducted acutely, thus the procedures are non-survival. This acute approach enables the direct manipulation and measurement of brain circuits without the additional

complication of performing these techniques in behaving animals (needs no chronic implantation of tools into brain/skull and warrants less noisy recordings due to a better controlled environment and the absence of movement artefacts), and thus allows faster and more efficient testing of hypotheses regarding how brain regions of interest interact.

Neuro-intervention and neuro-measurement in anesthetized animals: Brain activity will be manipulated (excitation or inhibition) at the neuronal or network level using pharmacology, optogenetics, pharmacogenetics, deep-brain stimulation (DBS) or by performing lesions (see 3.4.4.2). These techniques can facilitate or disrupt the activity of a group of neurons in a local region (e.g., optogenetics), neurotransmitter systems or entire brain networks (e.g., DBS). Such interventions will allow us to establish causal relationships between neural correlates of interest, which is one of the key aims of this proposal. For these experiments, we will measure the difference in neuronal responses between a baseline time when the manipulation had not been performed and following this intervention. Measurements will be collected using neurobiological activity using calcium imaging, electrophysiology, electrochemistry, microdialysis, and fMRI (see 3.4.4.3).

"Neuro-measurement" techniques to measure brain activity in anesthetized rodents (techniques previously described in **3.4.4.2**) are:
Measure-1) electrophysiology to assess neuronal firing and brain network activity
Measure-2) electrochemistry to assess fast neurotransmitter release (e.g., fast-scan cyclic voltammetry)
Measure-3) microdialysis to assess slow neurotransmitter release
Measure-4) calcium imaging to assess neuronal ensemble activity

Both neuronal activity and neurotransmitter release are studied and measurements focus on both local and global processes. We need such an array of measurement techniques to increase the chance that we can identify the neurobiological correlates of the behavior studied and thus find targets for subsequent intervention experiments (3.4.4.3), as well as targets to perform subsequent measurement experiments (3.4.4.2), or the combination of the two (3.4.4.4).

"Neuro-intervention" techniques to measure brain activity in anesthetized rodents (techniques previously described in **3.4.4.3**) are:
Intervent-1) Deep-brain stimulation (DBS)
Intervent-2) pharmacogenetics
Intervent-3) optogenetics
Intervent-4) lesions
Intervent-5) pharmacological treatments

One "neuro-measurement" technique will be combined with one "neuro-intervention" technique.

Neuro-intervention and neuro-measurement in brain slices: After sacrificing the animal and collecting the brain, neuronal activity will be manipulated in brain slices using pharmacology, optogenetics, pharmacogenetics, or by DBS (see 3.4.4.2). Such interventions will allow us to establish causal relationships between neural correlates of interest. Measurements will be collected using neurobiological activity using calcium imaging, electrophysiology, and electrochemistry (see 3.4.4.3).

In a subset of animals, we will perform this procedure (3.4.4.5) at the end of a pilot study/experiment carried out under a different procedure (up to 25% of the animals from 3.4.4.1; and potentially in a small set of animals from 3.4.4.2, 3.4.4.3, and 3.4.4.4) to either reduce the number of animals needed in our project or to assess the effects of previous experience on brain function. In either scenario, no discomfort would be added.

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

This procedure can consist of the following steps:

1. Animals are housed together until they become at least young adults (8 weeks of age). Then they are handled and weighed frequently.
2. (optional) In case of calcium imaging (Measure-4), a virus (e.g., AAV) that will express proteins that make calcium fluorescent and thus optically detectable, is infused into the brain via stereotactic microinfusion (under proper anesthesia (e.g., isoflurane) and perioperative analgesia; at least 3-4 weeks recovery from this surgery to allow the virus to express). Similarly, in case of

pharmacogenetics (Intervent-2) and optogenetics (Intervent-3), a virus that will express proteins that will make infected neurons sensitive to pharmacological (e.g., clozapine-N-oxide) or optical (e.g., light-sensitive so-called opsins) treatment, is infused.

3. For the anesthetized experiments: Measurement devices are acutely lowered into the animals' brains through holes that are drilled into the skull (under proper anesthesia (e.g., urethane) and perioperative analgesia). Some the equipment will be anchored onto the animals' skull with screws and dental cement. Depending on the technique, the devices consist of electrodes (Measure-1 and Measure-2), a guide cannula to enable lowering of electrodes (Measure-2) or a semipermeable membrane (Measure-3), fiber optics (Measure-4), or a post for head fixation (Measure-4). During the same surgery, intervention devices are lowered into the animals' brains. Some of the devices will be anchored onto the animals' skull with screws and dental cement. Depending on the technique, the devices consists of electrodes (Intervent-1), a guide cannula to enable the infusion of pharmacological agents (Intervent-2, Intervent-4, and Intervent-5), or fiber optics (Intervent-3).
   For the brain slice experiments: Animals will be sacrificed and the above mentioned measurement and intervention techniques will be applied in brain slices.

Testing of females. When we use female animals, estrous cycle may be checked frequently to control for potential sex hormonal effects on the brain and to determine when to conduct crucial parts of the experiments (e.g., experiments on females should all take place in the same period of the estrous cycle to prevent divergent effects of sex hormones on the brain). A small subset of female animals (under proper anesthesia and perioperative analgesia) is ovariectomized to control for variability due to estrous cycle. Surgery and recovery: 1 week.

After completion of the collection of data, the animals will be sacrificed (overdose of Nembutal and perfused for brain fixation) and their brains will be collected for histology and immunohistochemistry (e.g., stains to confirm the localization of the electrodes/other devices, stains to assess viral expression, and/or stains to assess the effects of "neuro-intervention" (e.g., electrode stimulation)). In the case of electrophysiology and voltammetry a small electrolytic lesion under continued anesthesia will precede the Nembutal treatment and perfusion (animal is still under proper anesthesia; no additional discomfort for the animal). Thus, animals will not recover from surgery/anesthesia.

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

Pilot experiments: Establishing new or adapted procedures requires step-by-step introduction and adaptation on the basis of obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Qualitative analysis: when experience with a certain test is limited to pilot experiments or indicates high variability, the number is based on the pilots and on literature data.

Quantitative analysis: when experience allows the calculation of numbers of animals to obtain a certain effect with statistical significance, we perform a power analysis to ensure that we use the minimum number of animals per group that will be statistically sound and biologically relevant.

B. The animals

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

Species used:

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

Rats (rattus norvegicus): genetically modified and wild type; rats are obtained from our own breedings or from a commercial licensed breeder.

Rats and mice are the best investigated mammal species used for fundamental research with significant knowledge about the anatomy and physiology of the rodent brain. The latest, most sophisticated technologies for investigating brain mechanisms are made for use in these species, including a variety of genetically engineered strains. It is required to use both strains because each strain offers specific advantages. Rats exhibit a greater spectrum of complex behaviors that are essential for assessing compulsive behavior and its components (and some genetic tools are available for rats). In addition,

measurement techniques are more widely available and more easily applicable in rats.

In contrast, many genetic tools are available for the manipulation of neuronal activity in mice (but mice exhibit a narrower spectrum of complex behaviors). The use of mice in addition to rats is mainly based on the availability of transgenic mice showing increased spontaneous grooming (no additional pharmacological treatment or behavioral training is required), such as the Sapap3-mutant mouse, which has been validated as an animal model for obsessive-compulsive disorder. Another factor is the possibility to study individual differences, where e.g. the fact that we breed transgenic mice (such as Sapap3-mutants) ourselves provides a natural opportunity to study individual differences.

Sex used: We aim for efficient use of both males and females from the animal lines that are bred in-house. In most other cases, males are used as they present the standard sex in the literature and almost all reference protocols and publications are based on the use of male rodents. Up to now, the overwhelming majority of behavioral and physiological studies on compulsivity in animals was carried out in male rodents. However, sex differences in clinical compulsivity have been reported. We plan to evaluate the experience of studying sex differences and decide if using female rodents in other parts of this project would be of scientific value. Since we aim for an efficient use of both males and females from the animal lines that are bred in-house, in some cases both males and females are used in the same experiment. In case sex differences become foucs of an experiment, it is necessary to use males and females in the same conditions and during the same time period to be able to properly compare them.

Animal number: All animals will be young adults or adult at the start of the experiments. The estimate of the total number of experimental groups is primarily based on our experience over the past years with the introduction of new paradigms and techniques. Thus, there are some factors involved that cannot be determined precisely. However, in general, an estimate for the total number of rats and mice is as follows: Neuro-intervention/measurement studies in anesthetized animals (3.4.4.5) contain an average of 20 animals (experimental group plus controls). Based on the present plans, we will use 1100 animals in this appendix, 400 mice and 700 rats. Approximately 75% of the animals will be exposed to moderate discomfort (recovery from stereotactic surgery for injection of virus or pharmacological agents), and the remaining 25% will experience mild discomfort. Approximately 75% of the animals will be used in the anesthetized preparation, and the remaining 25% will be used for *in vitro* slice experiments (discomfort does not differ between the two because both procedures are non-survival).

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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

☒ No

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

D. Replacement, reduction, refinement

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

These types of studies are conducted in both rats and mice worldwide, making translation and extrapolation of data between research-groups feasible.

Furthermore, the procedures described in this project are based on a large body of scientific- and experimental experience in both rats and mice. It is necessary to use both species because each of them offer specific advantages: Rats have a greater range of complex behaviors enabling better assessment of cognitive functions; more genetic tools and mutants are available for mice and one of our most important animal models is a mutant mouse strain.

Experiments will be executed in succession and, if needed, small explorative studies will be performed to provide the necessary insight in variation and expected results. On basis of this previous work and

experience, statistical analysis can be performed to determine the maximum number of animals needed to obtain interpretable data.

In principle we will use both male and female rats and mice. In particular in the case of the (transgenic) animals that are bred in house, this will lead to a reduction of "breeding surplus".
Some animals from 3.4.4.1 (up to 25% of 3.4.4.1 animals; but probably significantly fewer) and in small numbers from 3.4.4.2, 3.4.4.3, and 3.4.4.4 will be transferred to 3.4.4.5 for further non-behavioral experimentation to reduce the total number of animals used for our project.

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

All surgical procedures resulting in animal suffering or pain will be performed under adequate anesthesia (and analgesia).

In case of stereotactic intracerebral injections of viruses or other agents (75% of the animals) prior to the experiment, close postoperative monitoring will be performed and clearly defined humane endpoints applied. Animals will be allowed to recover from surgery for one week. All available resources to reduce pain, fear or suffering will be employed.

Procedures will only be performed by competent personnel, as mandatory.

Adverse environmental effects are not present.

Rats and mice will be socially housed and provided with environmental enrichment.

Repetition and duplication

E. Repetition

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

The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

Adequate anaesthesia and analgesia is used for all procedures.

I. Other aspects compromising the welfare of the animals

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

Other aspects that may compromise the welfare of the animals are:

- In case of stereotactic intracerebral injections of viruses or other agents (75% of the animals) prior to the experiment, recovery from stereotactic surgery may lead to maximally moderate discomfort.

- Unforeseen surgical complications, such as excessive bleeding, adverse reactions to the applied anesthetic, or accidental severing of nerve fibers or blood vessels.

Explain why these effects may emerge.

Surgical procedures are subject to human error. These procedures cannot be executed with 0% failure rate and seldomly increased postoperative bleeding leads to maximally moderate discomfort.

There is considerable variability within rodent populations regarding the sensitivity to anesthetics and drugs.

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

J. Humane endpoints

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

☐ No > Continue with question K.

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

The maximum degree of cumulative discomfort in any combination of tests/measurements/interventions will not exceed moderate discomfort. Animals will be euthanized with pentobarbital (applied by i.p. injection), if:

1. Persistent weight reduction (i.e., 20% or more compared to the weight at the experimental start in animals fed ad libitum and 10% in food-restricted animals), or acute weight loss within 2 days (15% in animals fed ad libitum and 10% in food-restricted animals) leading to more than moderate discomfort.
2. Abnormal behavior and/or posture, immobility, dirty fur, and other signs of distress, sickness, other unexpected circumstances leading to more than moderate discomfort.
3. Open wounds in Sapap3-mutant mice leading to more than moderate discomfort (10-20 % of older (> 6 months) mice; almost none in younger Sapap3-mutants).

Indicate the likely incidence.

Humane endpoints are expected to be met in 0-5 % of the animals tested within time frame of the experiments.

K. Classification of severity of procedures

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

Level of discomfort: Most animals (~ 75%) will receive an intracranial injection during a stereotactic surgery prior to the above described non-survival procedure. The recovery of this surgery is deemed moderate discomfort (for one week). Thus, we estimate up to 75% of the animals to experience a period of moderate discomfort for up to one week after stereotactic surgeries; but not discomfort otherwise

because these experiments are non-survival.

In total, we estimate that of the 400 mice, 300 will experience a period of moderate discomfort for up to one week after stereotactic surgeries; the remaining 100 will experience mild discomfort.

Of the 700 rats, 525 will experience a period of moderate discomfort for up to one week after stereotactic surgeries; the remaining 175 will experience mild discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

Rats and mice will be killed for histological and immunohistochemical analyses.

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

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

☒ Yes

principal approach

additional approach

3.4.4.1 establishing and characterizing rodent behavior that models compulsive behavior and its components
main read-out: **behavior**

models of compulsive behavior,

- induced by:
  - drug self-administration
  - genetic modification
  - pharmacological treatment
  - optogenetic stimulation
  - behavioral conditions
- characterized by:
  - escalation & persistence despite negative consequences

are tested for components:

- habit-formation
- cognitive flexibility
- fear and anxiety
- aggravation by stress , alleviation by environmental enrichment

3.4.4.2 identification of brain correlates of compulsive behavior and its components
main read-out: **behavior** & **neuronal activity**

behavioral testing (see box on the left)

plus neuro-measurement
1) electrophysiology
2) electrochemistry
3) microdialysis
4) calcium imaging
5) fMRI

3.4.4.3 establishing causality between brain pathways and compulsive behavior and its components via brain manipulation
main read-out: **behavior**

behavioral testing (see box on the left)

plus neuro-intervention
1) deep-brain stimulation
2) pharmacogenetics
3) optogenetics
4) lesions
5) pharmacology

3.4.4.4 establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation
main read-out: **behavior** & **neuronal activity**

behavioral testing (see box on the left)

plus neuro-measurement & neuro-intervention

1 measurement technique + 1 intervention technique

3.4.4.5 identification and characterization of neuroanatomical connections and their regulation
main read-out: **neuronal activity**

neuro-measurement & neuro-intervention in anesthetized animals 1 measurement technique + 1 intervention technique

Project [redacted] - AVD-801002015126 - Nederlands Herseninstituut-KNAW
Neurobiology of compulsive behavior and its components: Brain stimulation and measurements

	Procedure			mild	moderate
3.4.4.1	**Behavioral testing only**	Group 1a	rats	300	
		Group 1b	rats		700
		Group 1c	mice	150	
		Group 1d	mice		350
3.4.4.2	**Behavioral testing plus neuro-measurement**	Group 2a	rats		700
		Group 2b	mice		350
3.4.4.3	**Behavioral testing plus neuro-intervention**	Group 3a	rats		700
		Group 3b	mice		350
3.4.4.4	**Behavioral testing plus neuro-measurement and neuro-intervention**	Group 4a	rats		150
		Group 4b	mice		150
3.4.4.5	**Neuro-measurement and neuro-intervention in anesthetized animals**	Group 5a	rats	175	
		Group 5b	rats		525
		Group 5c	mice	100	
		Group 5d	mice		300
Total rat		**3250**		475 14.6%	2775 85.4%
Total mice		**1750**		250 14.3%	1500 85.7%

Format DEC-advies

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

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD/801002015126
2. Titel van het project: Neurobiology of compulsive behavior and its components: Brain stimulation and measurements.
3. Titel van de NTS: Compulsief gedrag en zijn componenten: neurobiologische metingen en hersenstimulatie
4. Type aanvraag:
   - ✓ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer
5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ✓ ontvangen door DEC: 19-06-2015
   - ✓ aanvraag compleet (herziening) ontvangen: 13-07-2015
   - ✓ in vergadering besproken: 29-06-2015
   - ✓ anderszins behandeld: n.v.t.
   - ✓ termijnonderbreking(en): n.v.t.
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ✓ advies aan CCD: 15-07-2015
7. Eventueel horen van aanvrager
   - Datum: n.v.t.
   - Plaats: n.v.t.
   - Aantal aanwezige DEC-leden: n.v.t.
   - Aanwezige (namens) aanvrager: n.v.t.
8. Correspondentie met de aanvrager:
   - Datum 30-06-2015
   - Strekking: suggesties voor completering van de aanvraag
   - Datum antwoord (gecompleteerde versie): 13-07-2015
   - Strekking van de antwoorden: de aanvraag is gecompleteerd
9. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

B. Beoordeling (adviesvraag en behandeling)

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

C. Beoordeling (inhoud):

1. Het project is wetenschappelijk verantwoord.

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

3. De doelstelling en de uitvoering om de doelstelling te bereiken is door de indiener duidelijk omschreven in de aanvraag: Het met behulp van dierexperimenteel werk verkrijgen van fundamenteel-wetenschappelijke neurobiologische inzichten in het ontstaan van compulsief gedrag en in de verschillende componenten die ten grondslag liggen aan compulsiviteit. Het gebruik van de bestaande proefdiermodellen voor dwangmatig gedrag en de verdere ontwikkeling van nieuwe modellen zullen een beter inzicht geven in neuronale basis van een breed scala aan neuro-psychiatrische ziektebeelden in de mens zoals obsessief-compulsieve persoonlijkheidsstoornis (OCD), verslavingsgedrag en eetstoornissen.

   Het fundamenteel wetenschappelijke belang van het project acht de DEC substantieel: Compulsiviteit is betrokken bij een grote verscheidenheid aan gedragingen en afwijkingen in (componenten van) compulsiviteit en kunnen leiden tot ingrijpende gedragsstoornissen. Het verkrijgen van fundamentele wetenschappelijke kennis van de neuronale mechanismen die ten grondslag liggen aan compulsiviteit is van belang voor een beter inzicht in het functioneren van het gedrag van de mens. Bij het ontstaan van compulsiviteit-stoornissen zijn verschillende componenten betrokken zoals stress en angstgevoelens, de mate van gewoontevorming en het verlies van controle over doelgericht gedrag. Een betere onderbouwing van de hypothese dat de verschillende componenten en uitingen van compulsief gedrag gereguleerd worden door dezelfde of overlappende neurale circuits is van klinisch belang omdat ontregeling van deze circuits mogelijk een gemeenschappelijk oorzaak vormt voor uiteenlopende neuro-psychiatrische ziektebeelden. Inzicht in de manier waarop de verschillende hersengebieden in deze circuits bijdragen aan de regulering van (componenten van) compulsief gedrag zal niet alleen bijdragen aan een beter begrip van compulsiviteit-stoornissen in de maatschappij, het biedt ook kansen om deze stoornissen te corrigeren en bestaande therapieën (m.n. diepe hersenstimulatie) te verbeteren. Het project dient daarmee, op termijn, een belangrijk maatschappelijk belang.

4. De gekozen strategie, experimentele aanpak in combinatie met de infrastructuur op het Nederlands Herseninstituut en de expertise van de betrokken onderzoeksgroep bieden

een realistisch uitzicht op het behalen van de beoogde doelstellingen binnen gevraagde looptijd van 5 jaar van het project. De onderzoeksgroep is ingebed in een grote klinische onderzoeksgroep van de afdeling psychiatrie van [redacted], hetgeen de uitwisseling van nieuwe kennis en inzichten tussen kliniek, klinisch onderzoek en het dierexperimentele werk in grote mate bevordert. Het reeds verrichte onderzoek van de groep heeft al belangrijke resultaten en publicaties opgeleverd en vormt een goede basis voor het voorgenomen onderzoek. Het gebruik van invasieve technieken met een hoge temporele en spatiële resolutie, om zo inzicht te krijgen in de relatie tussen neuronale activiteitspatronen en compulsief gedrag, is niet mogelijk in de mens. Beide onderzoekslijnen zullen naast elkaar worden uitgevoerd met een sterke onderlinge wisselwerking. Het dierexperimenteel onderzoek richt zich primair op een drietal verschillende diermodellen die reeds in het lab aanwezig zijn maar in het kader van het project zal ook worden onderzocht of andere modellen kunnen toegevoegd om zo de verschillende componenten van compulsief gedrag in optimale modellen te kunnen onderzoeken.

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

6. Het cumulatieve ongerief gepaard gaand met de dierproeven, zoals beschreven in de vier verschillende type dierproeven, is naar inschatting van de DEC, voor het merendeel van de dieren matig (85% van de 1750 muizen en de 3250 ratten) en voor de overige dieren licht. Deze inschatting van de DEC is volledig in overeenstemming met het niveau van cumulatief ongerief zoals dat is geclassificeerd door de onderzoekers. Hun classificatie is gebaseerd op hun ervaring met de gebruikte modellen in vergelijkbare, al uitgevoerde, dierproeven.
   Er moet worden opgemerkt dat in sommige modellen het noodzakelijk is om aversieve stimulaties (bijvoorbeeld pijnlijke korte elektrische schokken) te gebruiken als onderdeel van de gedragstesten en dat pijnbestrijding in deze gevallen niet wordt toegepast omdat dit strijdig is met de doelstelling van het experiment. In alle andere gevallen wordt adequate pijnbestrijding gebruikt.

7. Binnen het project wordt maximaal gebruik gemaakt van methoden die de voorgestelde dierproeven geheel of gedeeltelijk **vervangen.**
   Een belangrijk onderdeel van de experimentele strategie is de wisselwerking tussen gedragsstudies en klinische (interventie)studies bij de mens en het dierexperimenteel onderzoek. De klinische resultaten zullen worden gebruikt om een gerichte keuze te maken uit een groot aantal mogelijke startpunten van het dierexperimenteel werk.
   Voor het verkrijgen van nieuw inzicht in basis van compulsiviteit gestuurd gedrag is onderzoek op neuronaal activiteitsniveau essentieel. Met de huidige stand van de techniek kan dit type onderzoek met een sterk invasief karakter niet (of slechts bij hoge uitzondering) in proefpersonen worden uitgevoerd. Naar het oordeel van de DEC zijn er geen alternatieven beschikbaar voor het voorgestelde gebruik van intacte dieren om te doelstelling van dit project te realiseren.

8. In het project wordt optimaal tegemoet gekomen aan de vereisten van **vermindering** van dierproeven. Het gebruik van zowel mannelijke als vrouwelijke dieren uit de fok draagt bij aan een reductie van aantal dieren gedood in voorraad maar kan in sommige gevallen ook de variatie in de metingen verhogen waardoor er wat meer dieren ongerief zullen ondervinden. Inzicht in sekseverschillen is echter ook onderdeel van de vraagstelling.
   De onderzoeksgroep heeft veel ervaring met dit type experimenten. Een belangrijk onderdeel van de experimentele strategie is de gefaseerde opzet zoals beschreven in onderdeel 3.4.3 en gevisualiseerd in de bijlage “flow chart”. Eerst wordt een nieuw model en de benodigde methoden om betrouwbare uitleesparameters met een zo laag mogelijke variabiliteit te behalen volledig geoptimaliseerd. Daarna wordt overgegaan tot vervolgexperimenten om (i) de neuronale activiteit gekoppeld aan het gedrag te bestuderen of (ii) een interventie strategie te bestuderen. Pas daarna zullen de neuronale activiteitsbepalingen en de interventies in een enkel dier worden bestudeerd. Op die manier wordt een empirische cyclus doorlopen, waarbij kennis uit voorgaande proeven leidt tot een optimaal design van de vervolgexperimenten, waardoor per experiment telkens niet meer dat het minimum aantal benodigde dieren wordt ingezet.
   Technieken en procedures worden zorgvuldig toegepast. Het totaal aantal te gebruiken dieren in het project is een realistische schatting, mede gebaseerd op de aantallen dieren gebruikt in het verleden in vergelijkbare experimenten.

9. De uitvoering van het project is in overeenstemming met de vereisten van **verfijning** van dierproeven en is zo opgezet dat de dierproeven met zo min mogelijk ongerief worden uitgevoerd.
   Bij de opzet wordt rekening gehouden met dierenwelzijn en wel op de volgende manieren: 1) het gebruik van adequate anesthesie en analgesie waar nodig/mogelijk, 2) toepassing van stress-verminderende procedures, 3) een intensieve monitoring van de proefdieren gecombineerd met duidelijk gedefinieerde humane eindpunten.

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

   Er is geen sprake van belangwekkende milieueffecten.

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

D. Ethische afweging

De centrale vraag voor de ethische afweging is of het belang van het doel van dit project opweegt tegen het ongerief dat de dieren ondergaan (geclassificeerd voor het merendeel van

de dieren als matig). Het doel van het project is het verkrijgen van fundamenteel wetenschappelijke inzichten in het ontstaan van compulsief gedrag en de verschillende componenten die ten grondslag liggen aan compulsiviteit. Het onderzoek is primair fundamenteel wetenschappelijk van karakter maar door een inbedding in een klinische onderzoeksgroep zijn bevindingen uit het dierexperimenteel werk ook direct toegankelijk voor een eventuele klinische toepassing. De verwachting is dat de resultaten van het onderzoek, op termijn, kunnen bijdragen aan een beter inzicht in de oorzaken van verschillende ziektebeelden waarbij een stoornis in compulsiviteit betrokken is. Het project dient daarmee, op termijn, *een belangrijk maatschappelijk belang*.

Het fundamenteel wetenschappelijke onderzoek in dit project is van hoge kwaliteit en het project is uit wetenschappelijk oogpunt verantwoord. De onderzoeksgroep beschikt over ervaring met de gekozen onderzoeksstrategie en met de voorgestelde typen dierproeven. De DEC is van mening dat de resultaten van de dierproeven zullen bijdragen aan het behalen van de geformuleerde doelstellingen en schat de kans op het realiseren van deze doelstellingen in als hoog. De verkregen fundamenteel wetenschappelijke kennis is onmisbaar om te komen tot een beter begrip van de neurobiologische mechanismen die een rol spelen in compulsiviteit en het project dient daarmee een *substantieel wetenschappelijk belang*.

Bij het uitvoeren van de dierproeven wordt een adequate invulling gegeven aan de vereisten op het gebied van vervanging, vermindering en verfijning van de dierproeven. De DEC onderschrijft dat de doelstellingen niet zonder het gebruik van proefdieren kunnen worden behaald.
De DEC merkt op dat het een aanvraag betreft met de inzet van een groot aantal verschillende modellen voor compulsiteit en componenten van compulsiviteit gekoppeld aan verschillende meetmethoden van neuronale activiteit en methoden voor interventie. Dit schept een situatie waarbij een groot aantal verschillende combinaties mogelijk zijn. De DEC onderkent dat het voorgestelde onderzoek een sterk exploratief karakter heeft waarbij het moeilijk in te schatten is welke van de combinaties de grootste wetenschappelijke opbrengst zullen hebben. De randvoorwaarden zijn naar de mening van de DEC voldoende duidelijk vastgelegd zodat een ethische afweging mogelijk is. De IvD zal scherp moeten toezien dat de ingediende studieprotocollen binnen de afbakening van het projectvoorstel blijven.

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

E. Advies

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

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Bijlagen
2

Datum 15-07-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning dierproeven

Geachte heer [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 15 juli 2015.
Het aanvraagnummer dat wij hieraan hebben gegeven is AVD801002015126. Gebruik dit nummer als u contact met ons opneemt.

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

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

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:

- Gegevens aanvraagformulier
- Factuur

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD801002015126

Factuurdatum	15 juli 2015
Vervaldatum	15 augustus 2015
Factuurnummer	201570126
Betreft	Factuur Aanvraag projectvergunning dierproeven

Factuur

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD801002015126	€ 741,-

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

[redacted]

Van: secretariaat DEC [redacted]
Verzonden: woensdag 15 juli 2015 11:15
Aan: ZBO-CCD
Onderwerp: RE: indienen nieuwe PVA en CCD vergaderdatum en AVD-801002015126

Categorieën: [redacted]

Beste [redacted]

Nogmaals dank voor het doorgeven van deze nuttige informatie.
Ik heb zojuist alle documenten voor AVD-801002015126-[redacted] naar de CCD gestuurd via webftp. Het getekende aanvraagformulier wordt vandaag per post gestuurd.

Groet [redacted]
DEC-KNAW

From: ZBO-CCD [mailto:ZBO-CCD@minez.nl]
Sent: Monday, July 06, 2015 3:08 PM
To: secretariaat DEC
Subject: RE: indienen nieuwe PVA en CCD vergaderdatum

Beste meneer [redacted]

De optimale indiendata voor de verschillende CCD vergaderingen moeten nog door de CCD worden vastgesteld. Ik kan daarop vooruitlopend wel aangeven dat de optimale indiendatum voor de volgende CCD vergadering 15 juli is.
Ik hoop u hiermee voorlopig voldoende te hebben geïnformeerd.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.zbo-ccd.nl**
Postbus 20401 | 2500 EK | Den Haag
T: 0900 2800028
E: ZBO-CCD@minez.nl

Van: secretariaat DEC [redacted]
Verzonden: vrijdag 3 juli 2015 14:54
Aan: ZBO-CCD
Onderwerp: indienen nieuwe PVA en CCD vergaderdatum

Geachte CCD-medewerker,

Ik zou van u graag de uiterste PVA- inleverdatum ontvangen voor de volgende CCD bijeenkomst. Het verstrekken van deze informatie is op de laatste bijeenkomst toegezegd door de CCD. Op dit moment hebben we een aantal PVA in behandeling en willen graag iets meer zicht op een optimale indiendatum.
Ik hoor graag van u.

Groet [redacted]
DEC-KNAW

Dit bericht kan informatie bevatten die niet voor u is bestemd. Indien u niet de geadresseerde bent of dit bericht abusievelijk aan u is gezonden, wordt u verzocht dat aan de afzender te melden en het bericht te verwijderen.
De Staat aanvaardt geen aansprakelijkheid voor schade, van welke aard ook, die verband houdt met risico's verbonden aan het elektronisch verzenden van berichten.

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[redacted]

Van: [redacted]
Verzonden: maandag 20 juli 2015 13:46
Aan: ZBO-CCD
CC: [redacted]
Onderwerp: Re: Aanvraag AVD801002015126

Categorieën: [redacted]

To whom it may concern at the Centrale Commissie Dierproeven,

I would like to make sure that you are aware that the payment for our application AVD801002015126 is being submitted as soon as possible. Our finance department ensures us that it will be made by Friday, July 24.

Thank you.

Best,
[redacted]

[redacted]
Group Leader & Principal Investigator
Team [redacted] Royal Netherlands Academy of Arts and Sciences (KNAW) &
[redacted]

[redacted] Amsterdam
The Netherlands

[redacted]

From: ZBO-CCD <ZBO-CCD@minez.nl>
Sent: Wednesday, July 15, 2015 1:43 PM
To: [redacted]
Cc: [redacted]
Subject: Aanvraag AVD801002015126

Geachte heer [redacted]

Deze brief is ook per post verzonden.

Met vriendelijke groet,

Centrale Commissie Dierproeven www.zbo-ccd.nl Nationaal Comité advies dierproevenbeleid
www.ncadierproevenbeleid.nl .....
Bezuidenhoutseweg 73 | 2500 EK | Den Haag Postbus 20401 | 2500 EK | Den Haag
.....

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[redacted]

Van: Info-zbo
Verzonden: vrijdag 31 juli 2015 13:35
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD801002015126
Bijlagen: AVD801002015126_Vervolgbrief.pdf

Geachte heer [redacted]
Bijgevoegde brief is u vandaag ook per post verstuurd.

Met vriendelijke groet,

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**
Postbus 20401 | 2500 EK | Den Haag
Let op: vanaf nu heeft de CCD een nieuw e-mailadres info@zbo-ccd.nl. Heeft u ons oude e-mail adres in uw adressenboek, dan vragen we u om dat aan te passen.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD801002015126

Uw referentie
uw ref

Bijlagen
-

Datum 31-07-2015
Betreft Vervolg Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 15 juli 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neurobiology of compulsive behavior and its components: Brain stimulation and measurements" met aanvraagnummer AVD801002015126. Wij gaan uw aanvraag beoordelen. In deze brief leest u wanneer u een beslissing kunt verwachten.

Wanneer een beslissing
Wij nemen uiterlijk 08 september 2015 een beslissing. Omdat een DEC-advies is meegestuurd met de aanvraag, streven wij ernaar om de aanvraag binnen 20 werkdagen te beslissen.
Als wij nog informatie nodig hebben, kan dit later worden. Voor een complexe aanvraag staat een langere termijn. In beide gevallen ontvangt u daarover bericht. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Andere regelgeving
Hierbij wijzen wij u erop dat naast de regels die op de uitvoering van dierproeven van toepassing zijn op grond van de Wet op de dierproeven, er mogelijk ook verplichtingen kunnen voortvloeien uit andere wet- en regelgeving. In dit verband kan bijvoorbeeld worden gewezen op de Flora- en faunawet ten aanzien van in het wild levende dieren en de CITES-regelgeving ten aanzien van beschermde diersoorten.
Het besluit op uw aanvraag heeft alleen betrekking op de Wet op de Dierproeven en niet op andere wet- en regelgeving.

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

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Uw referentie
uw ref

Bijlagen
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Datum 31-07-2015
Betreft Vervolg Aanvraag projectvergunning dierproeven

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

Andere regelgeving
Hierbij wijzen wij u erop dat naast de regels die op de uitvoering van dierproeven van toepassing zijn op grond van de Wet op de dierproeven, er mogelijk ook verplichtingen kunnen voortvloeien uit andere wet- en regelgeving. In dit verband kan bijvoorbeeld worden gewezen op de Flora- en faunawet ten aanzien van in het wild levende dieren en de CITES-regelgeving ten aanzien van beschermde diersoorten.
Het besluit op uw aanvraag heeft alleen betrekking op de Wet op de Dierproeven en niet op andere wet- en regelgeving.

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

[redacted]

Van: [redacted]
Verzonden: maandag 10 augustus 2015 15:35
Aan: 'Info-zbo'
CC: [redacted]'
Onderwerp: RE: Aanvullende informatie aanvraag AVD801002015126
Bijlagen: 2. AVD-801002015126 NTS revised.docx

Beste [redacted]

Dank voor je verzoek voor aanvullende informatie m.b.t. de NTS. Een punt van aandacht voor toekomstige PVA stukken.
Namens de onderzoeker stuur ik als bijlage bij deze mail een herziene versie van de NTS.
Graag een bevestiging van ontvangst.

Groet [redacted]
[redacted] DEC-KNAW

From: Info-zbo <info@zbo-ccd.nl>
Sent: Monday, August 10, 2015 1:23 PM
To: [redacted]
Cc: Info-zbo
Subject: Aanvullende informatie aanvraag AVD801002015126

Beste Heer [redacted]
Zie bijgevoegde brief betreffende uw aanvraag AVD801002015126.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**
Postbus 20401 | 2500 EK | Den Haag
Let op: vanaf nu heeft de CCD een nieuw e-mailadres info@zbo-ccd.nl. Heeft u ons oude e-mail adres in uw adressenboek, dan vragen we u om dat aan te passen.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
Postbus 19121
1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

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

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Uw referentie
uw ref

Bijlagen
1

Datum 10 augustus 2015
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted],

Op 15 juli 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project “Neurobiology of compulsive behavior and its components: Brain stimulation and measurements” met aanvraagnummer AVD801002015126. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Niet technische samenvatting
De niet technische samenvatting bij uw aanvraag bevat een verwijzing naar appendix 1 (bij vraag 3.6). De NTS moet zelfstandig leesbaar zijn, daarom aan u het verzoek om deze verwijzing te verwijderen.
Graag ontvangen wij een nieuwe versie van de Niet technische samenvatting.

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

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

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

Met vriendelijke groet,

Datum
10 augustus 2015

Onze referentie
Aanvraagnummer
AVD801002015126

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:

- formulier Melding Bijlagen via de post

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

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

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

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

Aanvraagnummer	

2 Bijlagen

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

☐	
☐	
☐	

3 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

KNAW
[redacted]
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1000 GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
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info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD801002015126

Uw referentie
-

Bijlagen
1

Datum 12 augustus 2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 15 juli 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Neurobiology of compulsive behavior and its components: Brain stimulation and measurements" met aanvraagnummer AVD801002015126. Wij hebben uw aanvraag beoordeeld.

Op 11 augustus 2015 heeft u uw aanvraag gewijzigd. Op ons verzoek heeft u een kleine wijziging aangebracht in de Niet technische samenvatting, zodat deze op zichzelf leesbaar is.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. Gelet op het gegeven dat de aanvraag dierproeven bevat die op grond van de oude regelgeving nog uitgevoerd mocht worden en deze proeven (opnieuw) ter beoordeling zijn voorgelegd, mogen deze proeven alleen nog worden uitgevoerd onder deze door de CCD afgegeven vergunning.
U kunt met uw project "Neurobiology of compulsive behavior and its components: Brain stimulation and measurements" starten. De vergunning wordt afgegeven van 12 augustus 2015 tot en met 01 augustus 2020. De looptijd van de vergunning wijkt af van uw aanvraag omdat de startdatum op uw aanvraag in het verleden ligt.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC KNAW gevoegd. Dit advies is opgesteld op 15 juli 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Wij nemen dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering.
Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

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

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

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

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

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

Met vriendelijke groet,

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

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

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

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

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

deze projectvergunning voor het tijdvak 12 augustus 2015 tot en met 01 augustus 2020, voor het project “Neurobiology of compulsive behavior and its components: Brain stimulation and measurements” met aanvraagnummer AVD801002015126, volgens advies van Dierexperimentencommissie DEC KNAW.
De functie van de verantwoordelijk onderzoeker is Group Leader.
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 16 juli 2015
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 15 juli 2015;
   b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 11 augustus 2015 (herziene versie);
   c. Advies van Dierexperimentencommissie, ontvangen op 15 juli 2015

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst	Voorwaarden
Establishing and characterizing rodent behavior that models compulsive behavior and its component	Muis (Mus musculus) Rat (Rattus norvegicus)	500 muizen 1000 ratten	30% licht, 70% matig	Zie onder.
Identification of brain correlates of compulsive behavior and its components	Muis (Mus musculus) Rat (Rattus norvegicus)	350 muizen 700 ratten	matig	Zie onder
Establishing causality between brain pathways and compulsive behavior and its components via brain manipulation	Muis (Mus musculus) Rat (Rattus norvegicus)	350 muizen 700 ratten	matig	Zie onder
Establishing causality between putative brain correlates of compulsive behavior and its components and the behavioral readout via brain manipulation	Muis (Mus musculus) Rat (Rattus norvegicus)	150 muizen 150 ratten	matig	Zie onder
Identification and characterization of neuroanatomical connections and their regulation	Muis (Mus musculus) Rat (Rattus norvegicus)	400 muizen 700 ratten	300 muizen matig, 100 muizen licht. 525 ratten matig, 175 ratten licht	

Voorwaarden
Op grond van artikel 10a1 lid 2 Wet zijn aan een projectvergunning voorwaarden te stellen
De vergunning wordt verleend onder de voorwaarde dat eventuele go/no go momenten worden afgestemd met de IvD.

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

Er is enige overlap met een aantal al van een positief advies voorziene DEC protocollen.
Bij ingang van deze vergunning mogen de proeven die zijn beschreven in eerder van positief advies voorziene DEC protocollen alleen nog worden uitgevoerd onder deze door de CCD afgegeven vergunning.

Weergave wet- en regelgeving

Dit project en wijzigingen

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

Verzorging

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

Pijnbestrijding en verdoving

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

Einde van een dierproef

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

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

[redacted]

Van: Info-zbo
Verzonden: donderdag 13 augustus 2015 8:40
Aan: [redacted]
CC: [redacted]
Onderwerp: Beschikking AVD801002015126
Bijlagen: AVD801002015126_Beschikking.pdf

Beste heer [redacted]
Bij deze alvast per e-mail de beschikking betreffende uw aanvraag AVD801002015126.
Het origineel wordt u per post toegezonden.

M.vr.gr.

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**
Postbus 20401 | 2500 EK | Den Haag
Let op: vanaf nu heeft de CCD een nieuw e-mailadres info@zbo-ccd.nl. Heeft u ons oude e-mail adres in uw adressenboek, dan vragen we u om dat aan te passen.