	Inventaris Wob-verzoek W17-09								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS20171045								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel oud				x		x	x	
3	Niet-technische samenvatting	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	DEC-advies				x		x	x	
10	Ontvangstbevestiging				x		x	x	
11	Verzoek aanvulling aanvraag				x		x	x	
12	Reactie verzoek aanvulling				x		x	x	
13	Projectvoorstel nieuw				x		x	x	
14	Advies CCD		x						x
15	Beschikking en vergunning				x		x	x	

1.

06 APR 2017

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*	80100-KNAW; [redacted]
☐ 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	[redacted]

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	Groepsleider & Hoogleraar
Afdeling	[redacted]
Telefoonnummer	0 2 0 5 6 6 5 3 5 9
E-mailadres	[redacted]

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

(Titel) Naam en voorletters	[redacted] ☑ Dhr. ☐ Mw.
Functie	Groepsleider
Afdeling	[redacted]
Telefoonnummer	0 2 0 5 6 6 4 5 3 0
E-mailadres	[redacted]

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☑ 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	01-05-2017
		Einddatum	01-05-2027
3.2	Wat is de titel van het project?	Plasticity in the visual system and its regulation	
3.3	Wat is de titel van de niet-technische samenvatting?	De werking en regulatie van leren in het visuele systeem	
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]
		E-mailadres	[redacted]

4 Betaalgegevens

4.1	Om welk type aanvraag gaat het?	☑ Nieuwe aanvraag Projectvergunning € 1.827,00	Lege
		☐ Wijziging €	Lege
4.2	Op welke wijze wilt u dit bedrag aan de CCD voldoen. *Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.*	☐ Via een eenmalige incasso	
		☑ Na ontvangst van de factuur	

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht
☒ Projectvoorstel
■ Niet-technische samenvatting

Overige bijlagen, indien van toepassing
☐ Melding Machtiging
☒ Appendices 5 stuks; bijlage "overview number of animals"; bijlage "Figuren 1-4"

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	[redacted]
Functie	[redacted]
Plaats	Amsterdam
Datum	0 3 - 0 4 - 2 0 1 7
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'.	80100-KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	Provide the title of the project.	Plasticity in the visual system and its regulation

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.

Brain plasticity and its regulation

The brain shows a tremendous ability to adapt to its ever-changing environment. At the root of this adaptation is the formation and refinement of neural circuits (referred to as "plasticity"), allowing our brains to develop, acquire knowledge, learn new skills and recover from injuries.

The goal of this project is to increase our fundamental scientific understanding on how plasticity is accomplished. Our hypothesis is that plasticity in the brain is brought about and modulated by several interrelated mechanisms: i) rearrangements of feedforward connections (dominant at critical periods during development, ii) rearrangements of feedback connections (dominant in adulthood), iii) changes in the influence of interneurons to temporarily enhance plasticity so that it occurs only when necessary (for example: during specific stages of development or upon punishment or reward).

Connectivity of the visual system

To reach this goal, we use the visual system of the mouse as a model. The main reason is that there is a solid understanding of the basic wiring principles of the visual system, allowing us to study where and how changes in connectivity occur during plasticity.

The visual system responds to inputs from the two eyes (Fig. 1A). These feedforward inputs from the eyes first enter the lateral geniculate nucleus of the thalamus. Because a fraction of the axons from the retina do not cross in the optic chiasm, inputs from both eyes will enter the left and right thalamus. Thalamic relay neurons project to the primary visual cortex (V1). Neurons in V1 further process the information and send projections to higher visual cortices and thalamic nuclei in which more complex visual patterns are processed. The eyes also provide input to the superior colliculus which is important for regulating, among others, eye movements. The superior colliculus, in turn, projects to the thalamus. Within the visual system there are also extensive feedback connections (Fig. 1B). Visual cortical areas receive these feedback inputs from higher visual and frontal cortices providing contextual information about the visual scene, the task that is being carried out, the state of the animal, etc. The thalamus and superior colliculus also receive feedback information from the visual cortex. This reiterative connectivity makes it possible for the brain to anticipate and rapidly interpret the flow of information that enters the brain via the eyes.

Visual responses

When visual stimuli are provided to a mouse, neurons in the visual system will respond to it. This can be recorded using electrophysiological or imaging approaches. To what properties of visual stimuli a particular neuron responds depends on the brain region it is in and the specific synaptic inputs it receives. Neurons in the thalamus, for example, mainly respond to small patches on a contrasting background while neurons in V1 respond preferentially to bars moving in a particular direction. Neurons in V1 also have a preference for inputs from the left or right eye. This property is called ocular dominance (OD). It is a direct consequence of the fact that inputs from the two eyes project to both hemispheres. Binocular vision also enables depth perception, as it allows for the comparison of images from the two eyes. Surprisingly, mouse visual cortical areas including V1 do not only respond to visual stimuli but also to motor activity, reward or punishment, anticipation, decision making, etc. This is a consequence of the feedback inputs V1 receives from higher visual and prefrontal cortices and high order thalamic nuclei. In higher visual areas, neurons respond to more complex visual properties. Here, neurons may respond to particular objects or sceneries. Taken together, the responses in the visual system are defined by the circuits that the neurons form through their synaptic connections.

Plasticity in the developing and adult visual system and its regulation

Another reason why the mouse visual system is attractive for studying plasticity is that it is relatively easy to illicit and record responses in the visual cortex and subcortical regions upon visual stimulation and to relate changes in these responses (plasticity) to connectivity changes. Of special importance for our research is that there is a well-defined critical period of development during which plasticity of OD can take place. This allows us to compare the connections that undergo plasticity during this critical period, with the connections that undergo plasticity in adulthood. Moreover, it allows us to investigate the mechanisms that regulate plasticity during the critical period, and those that regulate adult plasticity, and relate these mechanisms to the specific connections that undergo plasticity.

i) Developmental plasticity and critical periods
During development, there are defined periods during which specific brain regions show a heightened plasticity potential. Generally speaking, lower order brain regions (for example those processing sensory inputs) undergo plasticity before higher brain regions (for example those involved in executive functions).
During the critical period of OD plasticity (Fig. 2, left panel) the feedforward connections from the eyes are fine-tuned which is important for the development of normal binocular vision. If misguided, for example by dysfunction or misalignment of one eye, the visual cortex will become less responsive to this eye causing the condition known as amblyopia, or "lazy eye". When in experimental animals one of the eyes is closed during a defined period of postnatal development, V1 will become less responsive to the deprived eye while responses to the non-deprived eye are strengthened. This functional change is accompanied by (and probably caused by) extensive structural changes: thalamic axonal feedforward projections from the deprived eye retract while those of the non-deprived eye expand. Notably, OD plasticity only occurs efficiently and permanently during a critical period of development (postnatal 3-5 weeks in mice). This has made OD plasticity very informative in the study of structure/function relationships during plasticity, and in understanding the mechanisms that regulate plasticity levels – specifically the factors that regulate the onset and closure of critical periods. We have used this model extensively, to identify molecular [1-5] and cellular mechanisms underlying and regulating critical period plasticity [6,7].

ii) Adult plasticity
In the adult visual system, after critical period closure, other mechanisms underlying plasticity become dominant. In adults plasticity is induced when large reductions in sensory input occur over prolonged periods of time. For example plasticity in primary visual cortex occurs after damage to the retina. This results in the "filling in" of the visual field so that one does not experience the lesion as a black spot. This form of plasticity is important, as like OD plasticity, it is induced by visual deprivation (though requiring an almost complete loss of input from (part of) the retina), but in contrast to OD plasticity it occurs readily in adulthood. This experimental model thus allows us to address the question whether it involves rearrangement of feedforward connections like critical period plasticity, or whether it alters feedback connections, as is expected for other forms of adult plasticity. In our laboratories we have employed this form of adult plasticity to study the relationships between the gain and loss of synapses and the trafficking of mitochondria [8].
A more natural type of adult plasticity occurs in association with perceptual learning: an improvement in the ability to detect or discriminate stimuli induced by repeated practice (Fig. 2 right panel). This is the type of learning that enables the trained birdwatcher to spot rare birds in the woods that untrained people miss out on. Perceptual learning takes place in adulthood and involves various brain areas including V1. In contrast to OD plasticity, perceptual learning is strongly modulated by reinforcement signals, such as reward or punishment [9]. With perceptual learning, responses of neurons in the visual system change. Interestingly, not only responses to particular features of trained visual stimuli alter, but neurons in the visual system also start responding to anticipated reward or punishment, choices the mouse makes or task-related behaviour. In our laboratories we have established perceptual learning tasks for mice, in which mice learn to differentiate between different visual stimuli. This lets us monitor, in real time, how the responses of hundreds of neurons change during learning. A related form of reinforcement learning is visual fear learning, during which a particular visual context is associated with an aversive stimulus such as an electric shock. Once the association is made, the animal will show freezing behaviour when the visual stimulus is presented to the mouse. These plasticity paradigms typically tune responses in lower visual brain regions to more complex contextual information. Therefore we believe these forms of plasticity involve plasticity of feedback connections providing such information.
Some forms of experience-dependent plasticity that occur readily in adulthood do not require reinforcement. This is called unsupervised learning and involves, for example, reduced responsiveness to repeated stimuli that initially provide a startling or novelty response. In our hands, mice learn not be afraid of objects that unexpectedly fly over or approach quickly, a process likely to involve interaction between the visual cortex and the superior colliculus. Another example it that mice stop paying attention to objects in their cage once they become familiar with them. Unsupervised learning is thus distinct from deprivation-induced plasticity, as it is induced by visual stimuli and not by continuous lack thereof, and from perceptual learning, as it does not require reinforcement. It is therefore important to understand

whether feedforward and feedback connections between thalamus, superior colliculus and V1 rearrange during this form of plasticity, and what the regulatory mechanisms are. Moreover, unsupervised learning will also occur (unintentionally) during reinforcement learning paradigms, as the mice will get used to the handling, the experimental setup, the visual stimuli that are shown repeatedly, etc. It is therefore important to know which changes in connectivity are induced by unsupervised learning in order to isolate those that are induced specifically by reinforcement learning.

Taken together, by studying and comparing how specific feedforward and feedback connections are reorganized during critical period plasticity, adult deprivation-induced plasticity, reinforcement learning and unsupervised learning we will be able to identify overarching principles of experience-dependent connectivity changes.

iii) Regulation of plasticity

From the above it is apparent that plasticity occurs at specific developmental stages, or under particular circumstances, for example upon reward or punishment or after prolonged lack of sensory input. This means that plasticity levels must be under regulatory control allowing the different forms of plasticity, such as critical period plasticity or perceptual learning to occur only when needed. A comprehensive understanding of the fundamental mechanisms of plasticity also encompasses an understanding of these regulatory mechanisms and how they relate to the changes in connections that effectuate plasticity under different circumstances. This can be achieved particularly well in mice, as many powerful approaches for genetic modification have been developed for mice, rapidly advancing our lines of research.

We and others have identified various molecular targets that regulate plasticity levels during the critical period. These include genes that regulate axon growth and retraction, synapse maturation or the formation of the extracellular matrix. Interestingly, many of these genes strongly affect plasticity levels when their expression is modified in a specific subset of inhibitory interneurons: parvalbumin (PV)-expressing basket cells. This strongly supports the idea that these interneurons play an important role in regulating plasticity levels during critical periods [6,7]. Interneurons represent 10-20% of all neurons in the brain. In contrast to excitatory neurons that employ glutamate as a neurotransmitter, interneurons use GABA as a neurotransmitter, through which they inhibit other neurons they contact. The onset of the critical period of OD plasticity involves the development of inhibitory innervation of the cortex [10] and thalamus [redacted] under revision for Nature Neuroscience). The further increase in the level of inhibition with development then appears to close the critical period, suggesting that a certain balance in inhibition and excitation is required for plasticity. Our work on a mouse model of Neurofibromatosis type 1 (NF1), a monogenetic developmental brain disorder associated with intellectual disability and autism, illustrates the relevance of mechanism. We find that in NF1 mice, cortical interneurons are hyperexcitable. As a consequence, these mice show early closure of the critical period. When these mice develop in an enriched environment, both cortical inhibition and critical period closure normalize. Importantly, PV+ interneurons provide important feedforward inhibition (they receive feedforward input, and inhibit excitatory neurons receiving the same inputs). Our hypothesis is that interneurons that provide feedforward inhibition are the perfect candidates to regulate plasticity of feedforward connections during critical periods.

Importantly, changes in inhibition/excitation balance also occur at the moment that plasticity is induced in adulthood. In the adult visual cortex, for example, inhibitory synapses are lost when the retina is damaged [11]. Moreover, the activity of interneurons in the visual cortex is reduced during perceptual learning [12]. These changes in inhibition appear to be crucial as activating or decreasing inhibition has been shown to reduce or increase plasticity levels [13,14]. Notably, the changes in inhibition that occur in adulthood seem to involve different subsets of interneurons than those regulating plasticity during the critical period (Fig. 2). Especially somatostatin (SST) expressing interneurons appear to be involved, which predominantly inhibit the dendrites of cortical neurons that receive feedback connections. We therefore hypothesize that interneuron subsets that gate feedback inputs are in the best place to regulate these connections in adulthood.

How the changes in the activity of inhibitory neurons or the persistence of their synapses are achieved remain unclear, as are the mechanisms through which disinhibition enhances plasticity during critical

periods or in adulthood. Our experiments have shown that during critical periods, inhibition does not have an instructive role, meaning that disinhibition does not selectively increase learned responses [7]. More likely, inhibition plays a permissive role, setting a level of plasticity. In summary, over the last years it has become clear that a temporary reduction of inhibition (disinhibition) is a critical factor in the enhancement of plasticity levels, not just during development but also in the adult visual cortex. However, this appears to involve different interneuron subsets, depending on the type of plasticity that occurs.

Open questions

Despite the growing knowledge on the anatomy of the visual system, very little is known about how experience-dependent plasticity is achieved through specific rearrangement of these connections. Most progress has been made in understanding the connectivity changes during OD plasticity during the critical period, where it is known that feedforward thalamocortical projections alter their connections to neurons in primary visual cortex. However, we have recently discovered that in contrast to what is generally believed, also within thalamus extensive OD plasticity takes place. This illustrates that even in this well-studied model, the basic principles are still not clear. What connectivity changes underlie perceptual learning, contextual fear learning or unsupervised learning in adulthood is even less well understood.

How plasticity levels are regulated is also not well understood. As explained above, disinhibition enhances plasticity. How disinhibition by specific interneuron subsets enhances plasticity, and how this can be achieved under the relevant circumstances and affect the relevant feedforward or feedback circuits needing adjustment remains unresolved. Moreover, the contribution of other mechanisms than disinhibition in the regulation of plasticity levels, such as axon growth and retraction, synapse maturation or the formation of the extracellular matrix, is unclear and requires further investigation.

The main goal of our research is therefore to gain fundamental scientific understanding on the general principles of how different types of plasticity are achieved through changes in feedforward or feedback connectivity and how these types of plasticity are regulated. We focus on the plasticity of the visual system as model.

This goal is of social relevance (see 3.3 for details). Since maladaptive plasticity mechanisms underlie a number of brain disorders (see below), understanding the overarching principles of plasticity mechanisms provides important future handles for studying the pathophysiology of such disorders. In addition, understanding the mechanisms through which plasticity levels are regulated may ultimately result in the development of approaches to enhance plasticity levels for therapeutic purposes, such as the treatment of neurodevelopmental disorders or improvement of recovery after brain damage.

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?

We aim to increase the knowledge on *the general principles of how different types of plasticity in the visual system are achieved through changes in feedforward or feedback connectivity and how these types of plasticity are regulated.*

To reach our main goal we formulated the following two sub-aims:

1. How are experience-dependent changes in brain function accomplished by rearrangements of neuronal circuits?

Our goal here is to define overarching principles of how different forms of plasticity (both developmental and adult) are achieved by modification of specific neural circuits. Our working hypothesis is that critical

periods involve rearrangement predominantly of feedforward connections, thus optimizing the circuits to process sensory inputs (Fig. 2). At later stages, associative/feedback connections are the dominant substrates of plasticity, allowing us to make novel associations. To test this hypothesis, we will identify the brain regions involved in critical period plasticity, perceptual learning, fear learning or unsupervised learning based on visual information, study the structural and functional properties of neuronal responses in these brain regions and how these change with learning, and compare the synaptic substrates of plasticity in these different developmental and adult forms of plasticity.

2. What are the cellular and molecular mechanisms that regulate plasticity levels?

Here we aim to understand how regulation of plasticity levels is achieved specifically in those brain regions and synaptic connections relevant for the learned task or function. A major focus will be on the role of disinhibition in this process. Our working hypothesis is that selective regulation of plasticity is achieved by specialized subsets of inhibitory neurons in the visual system. This hypothesis is supported by the finding that cortical (parvalbumin-expressing) interneurons that provide feedforward inhibition regulate plasticity of feedforward connections during the critical periods, while (somatostatin-expressing) interneurons that gate feedback connections regulate perceptual learning in adulthood (Fig. 2). To test this hypothesis, we will induce plasticity using defined plasticity paradigms, and compare how the activity of specific interneuron subsets and/or their synapses in the brain areas undergoing plasticity change. To establish a causal role of these changes in inhibition we will alter the activity of specific interneurons subsets or their synapses and study the effect on information processing and learning. It is also possible that specific signaling pathways are identified that regulate plasticity in defined brain regions or synaptic connections. We will therefore also use genetic or proteomic screening approaches and gene manipulation approaches to study molecular pathways regulating plasticity.

Expected outcome

We anticipate that at the end of this 5-year project we will have clearly defined whether our hypothesis that feedforward connections are the main substrate of critical period plasticity, and feedback connections are the main substrate in plasticity during adulthood is correct or not. We will also have tested whether these rules are limited to the primary visual pathway or whether they can be generalized to other (subcortical) brain regions involved in visual processing. We will also have shown which interneuron subsets regulate plasticity in thalamus and cortex during the critical period and in adulthood. We hope that we can also define roles for interneurons in other brain regions of the visual system, such as the superior colliculus. Finally, we anticipate that we will have identified molecular targets and signaling pathways that specifically affect plasticity of feedforward or feedback connections, or its regulation by specific interneuron subsets and may be relevant for understanding the pathogenesis or developing treatments for neurodevelopmental disorders such as neurofibromatosis type I.

Feasibility

The described research is highly feasible and represents ongoing and future research projects (see appendix "overview DEC proposals" for an overview of the approved DEC proposals that are currently in progress). Our laboratory is experienced and well equipped for performing the research lines. During the last 15 years we have been studying plasticity in the visual system and established a wide range of state-of-the-art experimental approaches enabling us to perform the planned research. These include experimental paradigms to induce plasticity during critical periods (OD plasticity) and in adulthood (lesion-induced plasticity, perceptual learning, habituation), approaches to record neuronal activity in vivo (single- and multiunit recordings, cell-attached recordings, optical imaging of intrinsic signal, in vivo two-photon calcium imaging) or in slices (multi-electrode patch-clamp recordings, field-potential recordings, calcium imaging), two-photon microscopy to chronically image synapse morphology in vivo, gene manipulation approaches (transgenesis, viral vectors, in utero electroporation etc.), immunohistochemistry, tissue clearing techniques, western blotting, etc. All of these techniques have resulted in publications in high impact journals [1,2,5,6,8,15-22] . Our research efforts are embedded in the environment of the NIN, providing excellent infrastructure, technological support and outstanding scientific interactions. Moreover, our research is well-linked within the national and international scientific

community.

The quality of our work is further underscored by the recognition through research funding agencies (e.g. NWO, HBP, EU). The planned research is currently funded by grants from NWO and the EU.

3.3 Relevance

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

Scientific relevance: Uncovering general principles of how neuronal networks can effectively improve their performance through experience will advance our scientific understanding on a fundamental neurobiological mechanism namely the ability of the brain to learn from experience. Our research is not only important for scientists working in the visual system, but for many scientists whose research is related to plasticity mechanisms in other brain regions. Our experiments also have impact on scientists working on artificial intelligence, brain-machine interfaces and neuroinformatics and for software developers making use of these approaches. Our laboratories perform research for the Human Brain Project, in which scientists building brain models rely on experimental information they receive from neuroscience laboratories. Moreover, the Human Brain Project incorporates the knowledge we acquire to develop robots with artificial intelligence.

Social relevance: Our research may, in the long run, have several important therapeutic implications. *First*, critical period plasticity in the visual system is an excellent model for studying the cause of amblyopia ("lazy eye") when vision is impaired during childhood due to misalignment of the optical axes or inequality of refractive power of the two eyes. Amblyopia is the most prevalent (2-4%) visual disorder in young people. Of all amblyopes, 3-18% will become visually impaired in their unaffected eye in the course of their life through injury or illness (like all people), causing binocular visual impairment and severe disability in 1:1000 people. The discovery of novel approaches to reactivate plasticity after critical period closure may result in novel avenues to treat amblyopia in adults.
Second, various neurodevelopmental disorders such as autism and intellectual disability, have been suggested to be caused by deficits in critical period plasticity. Understanding the principles of regulating plasticity levels may ultimately provide new handles on treating these diseases.

As an example: our work on mice lacking a copy of the Neurofibromatosis type 1 gene, causing autism and intellectual disability in humans, reveals that this deficit increases cortical inhibition and causes precocious critical period closure.

Third, finding approaches to (temporarily) increase cortical plasticity is also important for the development of regenerative and restorative therapeutic approaches. This is especially important for the future treatment of neurodegenerative diseases, improving brain function after physical trauma. In addition, various developments are underway for restoring vision in the blind. This includes reactivation of retinal function using optogenetics, but also implantation of electrode grids in the visual cortex to directly input visual information. These approaches will mostly likely require plastic changes to let the visual system adapt to the new types of the inputs.

The proposed experiments are primarily aimed at answering fundamental scientific questions on visual plasticity and the direct aim is not to develop new treatments. This notwithstanding, we have already found novel approaches to increase adult cortical plasticity and we are testing whether these can be applied to improve brain development in a mouse model of intellectual disability and autism (Neurofibromatosis type 1). This illustrates that our fundamental approach can lead to therapeutically relevant discoveries.

3.4 Research strategy

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

Sub-aim 1. How are experience-dependent changes in brain function accomplished by rearrangements of neuronal circuits?

This issue is addressed in different stages with decision points in between (see Fig. 3). Depending on the current knowledge on a particular form of plasticity or a particular brain region, projects may start at different stages.

A1. To determine how changes in neuronal connectivity result in functional plasticity, an appropriate paradigm is selected for inducing the form of plasticity of interest. The main categories of learning that we address and compare in the visual system are critical period plasticity (by eyelid suture) (Animal Procedures 3.4.4.1 and 3.4.4.2), lesion induced plasticity (by enucleation or retinal lesioning) (AP 3.4.4.1 and 3.4.4.2), perceptual learning (using reinforcement to train mice to respond to visual stimuli), contextual fear conditioning (by associating a visual stimulus with an aversive stimulus) and unsupervised learning (by repeatedly providing visual stimuli until the mouse is used to them) (all AP 3.4.4.3 and 3.4.4.4). The exact paradigm is selected based on the literature or our previous experience. In some cases, novel plasticity paradigms need to be developed, for example to study particular forms of perceptual learning or habituation.

A2. The next steps are aimed at determining the brain region in which the relevant information is processed (A2a) and plasticity occurs (A2b). In most cases, this is known from the literature and the brain region of study is selected together with the plasticity paradigm. However, for some types of plasticity it may be unclear where the relevant information is processed. For example, if a mouse performs a task in which it needs to learn to associate one image with a reward but not another image, one would first need to determine which visual areas are capable of differentiating between the two images. This means that if the function and anatomy of a particular brain region of the visual system is not sufficiently well-documented, we will need to investigate its function in more detail before we can examine its plasticity (AP 3.4.4.5). It may also be possible that experiments from the lab hint towards plasticity taking place elsewhere than always assumed. Our recent discovery that there is extensive OD plasticity within the thalamus illustrates this. Thus, in cases where the brain region of plasticity is not defined, recordings will need to be performed to identify and characterize regions in which a) the relevant information is processed and b) plasticity takes place. Depending on the brain region and specific plasticity type, our recordings can be done by two-photon microscopy of calcium signals, in vivo electrophysiology approaches or by optical imaging of intrinsic signal (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

Only if the relevant brain region is determined, the project is continued to study the nature of the underlying mechanisms leading to plasticity.

A3. We perform experiments to determine where in the circuitry of this brain area plasticity occurs. This analysis can take place at the anatomical or functional level. At the anatomical level this either involves chronic two-photon microscopy of fluorescently labelled neuronal structures, or post-hoc tracing experiments. At the functional level this may involve electrophysiological approaches or imaging approaches to identify the cell types/ synaptic structures that undergo plasticity. The focus will be on understanding whether changes in feedback or feedforward connections dominate and how functional and anatomical changes are related (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

A4. Finally, and if an experimental approach is available, we interfere with the observed plasticity in order to study the causal relationship (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

An example of our strategy: experiments in the lab suggested that OD plasticity may take place in thalamus. We chose to test this by establishing whether there were binocularly responsive neurons in thalamus. This was confirmed, and the nature of binocular responses was determined. Next it was decided to monocularly deprive mice during the critical period and study changes in binocular responses. We found that binocular neurons in the thalamus become more responsive to the open eye. To determine which inputs have altered (feedback, or feedforward), we plan to repeat the experiment, and measure whether the change in binocularity after deprivation remains the same when the visual cortex (which

provides feedback to thalamus) is silenced. If so, feedforward connections must be altered. Finally, to understand whether plasticity in V1 depends on plasticity in thalamus, we will inactivate plasticity genes (CamKinase II for example) in thalamus and study whether this interferes with plasticity in thalamus and V1

Sub-aim 2. What are the cellular and molecular mechanisms that regulate plasticity levels?

B1. To study how plasticity levels are regulated in a particular brain region, we first select the paradigm and brain region in which we want to address the question. This choice is made based on previous experiments (as described above) or from literature (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

B2. We will then study how cellular (inhibitory/excitatory balance for example) or molecular (gene/protein expression) properties change under these circumstances. We may study this at the anatomical level (change in synapse number) or functional level (change in synapse function or interneuron activity) or at the protein/gene expression level (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

B3. Finally, we will interfere with the change in inhibition in order to understand whether there is a causal relationship between the change in inhibition and plasticity and to study the mechanisms by which inhibition regulates plasticity. This will be accomplished by manipulating the activity of specific neuronal populations (opto/chemo-genetics). Candidate genes/proteins may also be selected (from B2) and their function altered in order to test how they regulate functional properties of the relevant brain region (AP 3.4.4.5) and what their role is in plasticity (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4).

Example: we knew from literature that inhibition regulates OD plasticity. We developed an approach to chronically visualize inhibitory synapses in mice. We used this approach to study the formation and loss of inhibitory synapses in V1 of mice during OD plasticity and discovered that they were rapidly lost during plasticity. To understand whether this relationship is causal, we will now interfere with the stability of inhibitory synapses by molecular intervention to study the causal relationship. We have also interfered with inhibitory interneuron function during the assessment of OD plasticity and found that changes in inhibition do not directly influence OD itself, but only its plasticity.

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.

The type of experiments we perform to address our research questions are very complex and have many experimental variables although they do not vary much in the degree of discomfort caused to the animals. It is therefore more effective to cluster the experiments based on whether plasticity is induced by visual deprivation (AP 3.4.4.1 and 3.4.4.2) or visual learning (AP 3.4.4.3 and 3.4.4.4), or whether it only involves characterization of the molecular/anatomical or functional properties of a brain region (AP 3.4.4.5) and on whether the readout is acute (AP 3.4.4.1, 3.4.4.3, 3.4.4.5) or involves chronic monitoring of neuronal function or structure and behaviour (AP 3.4.4.2, 3.4.4.4). All our experiments can be characterized by four different components (see Fig. 4):

1. Instrumentalization of the experimental animal.
2. The plasticity paradigm
3. The experimental manipulation
4. The readout

1. Instrumentalization of the experimental animal
Most of our experiments will require some type of gene modification in cells of the brain and/or cranial surgery in order to be able to use the mice for our experiments. Gene modification may be required to alter genes involved in (the regulation of) plasticity, to express proteins required for their visualization (for example by two-photon microscopy) or the modification of their function (for example by optogenetics or chemogenetics). The cranial surgery may be necessary for allowing the visualization of neurons (cranial window implant, or thinning or clearing of the skull), the implantation of a headpost for head-fixation, or the implantation of a recording chamber to allow the insertion of an electrode for

electrophysiological recordings.

Instrumentalization of the experimental animals can therefore involve the following (combination of) procedures:
1a) Breeding of wildtype, inbred and/or genetically modified mice.
1b) In utero electroporation: a limited number of neurons is genetically modified in the brain of embryos. This is achieved by opening the belly of an anesthetized pregnant female mouse, injecting DNA through the uterine wall into the brain ventricles of the embryos, and electroporating the DNA into the cells lining the ventricles. The muscle wall and skin of the belly are sutured closed again and the pups are born several days later, most of them expressing the electroporated DNA in targeted neurons of the brain.
1c) Transduction with viral vectors: a viral vector driving expression of a gene of choice is injected into the brain region to be studied, resulting in the widespread expression of the gene.
1d) Cranial surgery: for imaging experiments, a cranial window may be implanted above the brain region this is to be imaged. The skull can also be made translucent by thinning, or by applying nail polish. In both cases, a head post is attached to the skull allowing fixation of the head. For electrophysiology experiments, a head post and a recording chamber or electrode may be inserted allowing head-fixation and performing recordings.

Combinations of the different genetically modified mouse models are common. In many cases, viral vectors are used that are cre-dependent. These are injected in transgenic mice expressing cre in a particular brain region or cell type. Another example can be the use of in utero electroporation to express a receptor for a virus in a limit number of neurons, followed by transduction using a viral vector later in life that will selectively infect these neurons allowing the tracing of the synaptic inputs these cells receive.

2. Plasticity paradigms:
As explained above, different plasticity paradigms are used.

Visual deprivation paradigms (3.4.4.1 & 3.4.4.2):
2a) OD plasticity is induced by suturing one eyelid closed under anaesthesia. The eye remains closed for several days and is reopened before assessment of visual responses (see below).
2b) Retinal lesions can made in adult mice. This either involves binocular lesions in the binocular-projecting part of the retinas, or a monocular lesion in the monocularly projecting region (in order to eliminate all retinal input to the cortex responding to a particular region in the visual field). The lesion is made under anesthesia using a powerful laser. Lesion-induced plasticity can also be induced by enucleation under anesthesia.

Visual learning paradigms (3.4.4.3 & 3.4.4.4):
2c) Perceptual learning involves the training of mice in a particular task. The exact task will vary depending on the exact question and experimental readout (see below). In head-fixed tasks, mice first habituate to being head-fixed and in some cases, to walk on a treadmill without being fearful. Then they learn to associate a particular visual stimulus presented to them with a behavioural response (for example, lick left if a square is shown, lick right if a circle is shown). This training involves reinforcement, i.e. reward or in some cases, punishment. Reward usually consists of providing milk through a lickspout. Mice in such paradigms are water-restricted and get most of their fluid intake during the training. Head-fixation is required for many types of imaging or electrophysiology (see below). Head-fixation is not always necessary. In such cases, training may take place in a home cage or specialized environment.
2d) Visual fear learning involves pairing a particular visual context with an aversive stimulus. Upon conditioning, the mouse will show freezing behaviour upon seeing the conditioned visual stimulus.
2e) Unsupervised learning lets mice get used to visual stimuli that are novel or naturally perceived as threatening. A threatening stimulus can be a disc increasing in size projected on the top of the cage or a fly-over stimulus. These stimuli resemble approaching predators.
In many cases the paradigms will be carried out while the mice are head-fixed, allowing us to monitor or manipulate neuronal activity or morphology (see under 4) while plasticity is being induced.

3. The experimental manipulation
In order to causally test the involvement of a particular cell type or protein in the processing of relevant information or (the regulation of) its plasticity we use several approaches to interfere with the function of specific neurons or proteins.
To achieve this we use the following tools:
3a) Focal modulation:
We will locally stimulate or inhibit neural activity using transcranial, local and intracellular electrical stimulation and inactivation, and focal ultrasound delivery.
3b) Activation of designer proteins
We will activate designer proteins that have been expressed in the instrumentalization phase. We use different types of such proteins:
Optogenetics: after expressing light-sensitive proteins in specific neuronal subsets, their activity can be increased, decreased or more selectively modulated by shining light on them.
Chemogenetics: after expressing receptor proteins that bind a ligand that has little or no biological effect on other cells in the body, the ligand can be administered to the mouse resulting in the activation, suppression or more selective modification of neuronal function.
Ultrasound-mediated neuronal modulation: novel approaches for modifying neuronal activity are developing rapidly. One such development is the expression of mecanoreceptors that modify neuronal activity in an ultrasound -manner.
3c) (Opto)pharmacology: Pharmacology will be applied to alter neuronal activity or to target specific receptors. In some instances, Optopharmacology will be used (these are pharmacological agents that are activated by light).
3d) Housing conditions: By changing the conditions under which the mice are housed, plasticity levels can be manipulated. Environmental enrichment is known to alter the inhibition/excitation balance and to affect plasticity (this is illustrated by our finding that environmental enrichment reduces inhibition in NF1 mice and normalizes critical period closure in these animals). It has also been shown that rearing mice in complete darkness can keep the critical period for OD plasticity open. Housing adult mice in complete darkness can reopen the critical period.

4. The readout
The readout of neuronal activity in a particular brain region can be measured by various approaches. Each approach has its strengths and weaknesses, and which approach is used depends on the exact question and the brain region.

Acute assessment of molecular or neuronal properties using electrophysiology, histology or molecular analysis (3.4.4.1, 3.4.4.3, 3.4.4.5):
4a) In vivo probe recordings: this involves the introduction of an (multi-array) electrode into brain of the mouse and record electric activity of neurons, or an electrochemical probe for voltammetry. Before or during the recording session, the mouse is anesthetized, a hole is drilled in the skull and the electrode is inserted (see under 1d). Acute recordings are performed in awake or anesthetized mice and the animal is sacrificed immediately after completion of the recordings.
4b) Acute imaging of intrinsic signal or fluorescent indicators of neuronal activity. Optical imaging of intrinsic signal involves measuring the reflectance of red light shone on the brain. Changes in blood oxygenation and hemodynamics due to neuronal activity alter the light reflectance. For acute imaging, the scalp of the mouse is removed under anesthesia and the mouse is placed in the imaging setup and recordings take place for several hours while visual stimuli are presented.
Two-photon microscopy of neuronal activity: in these experiments two-photon microscopy is employed to detect changes in neuronal activity. To be able to detect neuronal morphology or activity, a fluorescent reporter is required. This can be a chemical reporter, which needs to be loaded into the brain before the onset of the recording. This is achieved by injection of the dye into the brain using a pipet while the mouse is under anesthesia. In most cases, however, genetically encoded reporters are used based on fluorescent proteins. Expression of these proteins thus requires gene transfection using viral vectors or in utero electroporation, and/or the use of transgenic mice. These procedures have to take place well before the measurement takes place. The recordings generally take place in head-fixed mice (see above), under anesthesia or in awake mice in which a cranial window has been implanted. A head stage is also implanted for fixating the head under the microscope or wide field imaging setup. Visual

responses are elicited by presenting visual stimuli to the mouse. Two-photon microscopy of calcium signals allows measuring the activity of hundreds to thousands of individual neurons in real time.
Wide field imaging of fluorescent markers for neuronal activity makes use of the same principles as two-photon microscopy, but at a different scale. This approach involves macroscopy and does not allow activity measurements at the single cell level, but at the population level. The advantage is that imaging of a much larger part of the cerebral cortex of the mouse can be imaged. Also for widefield imaging, the skull needs to be made translucent, either by the implantation of a cranial window or by application of agents rendering the skull translucent.
4c) Slice electrophysiology: mice are anesthetized and sacrificed, and the brain is rapidly removed, cooled and sliced. The brain slices can then be used to record neuronal/synaptic activity by electrophysiology or to acutely induce plasticity by electrical stimulation.
4d) Tissue extraction: to isolated RNA, DNA or proteins from tissue the mice are anesthetized and sacrificed, and the tissue is removed. In most cases the tissue is then snap frozen and stored for later extraction of the required molecular components.
4e) Histology: to collect tissue for histology, the mice are anesthetized and perfused with fixative. The tissue is then removed and post-fixed after which it is sliced and stained using immunohistochemistry. This approach is essential for anatomical and molecular assessment of brain tissue.
In many cases, combinations of the above will be used, such as histology after an in vivo electrophysiology experiment.

Chronic recording of neuronal activity and morphology (3.4.4.2, 3.4.4.4).
4f) In vivo probe recordings: (see 4a). Mice undergoing chronic recordings, have been implanted with a recording chamber or electrode (see 1d). The electrode may either be implanted chronically, or, alternatively, a recording chamber is implanted and the electrode is inserted only during the recording after which the whole is closed with bone wax. In both cases, the recording can be done repeatedly in awake, behaving mice or in anesthetized mice. This read-out may be followed by a final (acute) readout session described under 4a through 4e.
4g) Optical imaging of intrinsic signal (see above) can also be performed chronically. In this case skull clearing or cranial window implantation is performed as described above and a head-post needs to be implanted.
Two-photon microscopy of neuronal structure and/or neuronal activity: in these experiments two-photon microscopy is employed in the same way as described in 4b. However, the recording is performed repeatedly in awake or anesthetized animals, allowing us to detect changes in neuronal morphology (such as synapse size or density) or neuronal activity over prolonged periods of time. A cranial window and head-post is inserted as described under 1d. In case repeated imaging in deep structures of the brain is required, a guide tube for a GRIN lens is implanted in the brain, allowing chronic and repeated insertion of the lens.
Finally, wide field imaging of fluorescent markers for neuronal activity (see above) can also be performed chronically, in which case a head-post needs to be implanted. This read-out may be followed by a final (acute) readout session described under 4a through 4e.

Again, combinations of these techniques are often used: for example optical imaging of intrinsic signal to determine the retinotopy of V1 (i.e. how the visual field is processed within V1) followed by two-photon microscopy of fluorescently labelled neurons, or electrophysiological recordings of neurons that are fluorescently labelled for chronic imaging.

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.

This project includes all steps from developing plasticity paradigms, to identifying the relevant brain regions and neural substrates mediating and/or regulating learning, the testing of causal relationships, and investigating errors in neurodevelopmental disorders as described above.

The first subaim (Fig. 3A) is to compare how different forms of plasticity are mediated by changes in neural connectivity. In many cases, we will make use of established plasticity paradigms, such as OD

plasticity or lesion-induced plasticity. When we study circuit changes involved in altered behavior, we will adapt existing behavioral paradigms for perceptual learning, contextual fear conditioning or habituation in order to make them suitable for the automated tracking of behavior in combination with in vivo electrophysiology or imaging approaches. Once we achieve this for a particular form of visual plasticity we next identify the brain regions processing the relevant information. If identified (go/no-go) we will establish whether plasticity occurs in this brain region before continuing with detailed analyses (go/no-go). We then determine where in the circuitry of this brain region plasticity occurs, which will happen at both the anatomical and functional level by observing and testing where changes in connectivity or function occur. Finally, we interfere with the observed plasticity in order to study the causal relationship.

The second subaim (Fig. 3B) is to understand the cellular and molecular mechanisms that regulate plasticity levels. We will select a particular brain region and plasticity paradigm for which we want to address this question based on our previous results, obtained in subaim 3a, and the literature. We will assess changes in cellular or molecular properties during plasticity induction that are linked to plasticity regulation. Identified targets will be experimentally modified to validate a causal relationship and understand the underlying mechanisms.

It is important to note that there is overlap between the animal studies described in this project and those in earlier DEC-approved protocols and on which we are performing currently our animal experiments. After a license for this project has been obtained, all experiments will formally be executed under this new license. To illustrate the scientific topics we are working on, we included with this application a document entitled "*Overview current DEC protocols*". This also demonstrates that both the proposed Animal Procedures together play an essential role in obtaining our research objectives.

Reference List

11. Keck T., Scheuss V., Jacobsen R.I., Wierenga C.J., Eysel U.T., Bonhoeffer T., and Hubener M. (2011). Loss of sensory input causes rapid structural changes of inhibitory neurons in adult mouse visual cortex**.** Neuron *71***:** 869-882.
12. Makino H. and Komiyama T. (2015). Learning enhances the relative impact of top-down processing in the visual cortex**.** Nat. Neurosci. *18***:** 1116-1122.

13. Kuhlman S.J., Olivas N.D., Tring E., Ikrar T., Xu X., and Trachtenberg J.T. (2013). A disinhibitory microcircuit initiates critical-period plasticity in the visual cortex**.** Nature *501***:** 543-546.
14. Fu Y., Kaneko M., Tang Y., Alvarez-Buylla A., and Stryker M.P. (2015). A cortical disinhibitory circuit for enhancing adult plasticity**.** Elife. *4***:** e05558.

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	**Readout after visual deprivation:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual deprivation.
2	**Readout during visual deprivation:** Chronic in vivo assessment of neuronal function or anatomy in control mice and mice during plasticity induction by visual deprivation.
3	**Readout after visual learning:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual learning
4	**Readout during visual learning:** Chronic in vivo assessment of neuronal function or anatomy in control mice and mice undergoing plasticity induction by visual learning
5	**Readout in naïve mouse:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in mice in without plasticity induction
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'.	80100-KNAW	
1.2	Provide the name of the licenced establishment.	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 3.4.4.1	Type of animal procedure **Readout after visual deprivation:** acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual deprivation.

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 aim of this procedure is to measure the effect on neural structure and function by an experimental manipulation in mice after plasticity induced by visual deprivation.

The general design has the following components:

1. Instrumentalization of the experimental animal
2. Plasticity paradigm - visual deprivation
3. Experimental manipulation
4. Readout - acute session

We will first describe the specific aims of these components. The procedural details are given later following this section.

1. Instrumentalization of the experimental animal

Our experiments will use mice as experimental animal. We will use wild type animals (e.g. C57Bl/6), mouse strains carrying spontaneous genetic mutations (e.g. albino mice) and genetically modified mice to allow optical recording or to disturb the system. The genetic manipulation can have several aims:

- To express proteins, such as genetically encoded calcium indicators, that allow optical recording of neural activity
- To express fluorescent proteins, such as GFP, to label neurons for optical identification.
- To express light or ligand activated proteins, such as channelrhodopsin, halorhodopsin and DREADD, to influence neural function

- To knock-out, alter or express proteins that allow us to understand their role in information processing or plasticity in health and disease

We will use three ways to introduce genetic alterations.
1a. Transgenic mouse lines, without any additional experimental procedure.
1b. In utero electroporation, i.e. using animals in which vectors (including viral vectors) have been injected while they were embryos.
1c. Viral expression, i.e. using virus injections (juveniles – adults)
Procedures 1b and 1c may be happening in transgenic animals. To restrict the expression of genes to specific brain area, cell-types or period, we will make use of conditional expression techniques such as the cre-lox system. In addition to restrict the temporal expression we may use delivery of agents such as doxycycline. Any combination of procedures 1a, b and c may occur.

For most of our experiments it is necessary to do
1d. Cranial surgery to apply a head fixation post and create a cranial recording window or chamber.
We will head-fix the mice during some of the plasticity paradigms and recording of neural activity, because the presentation of sensory input, like visual stimuli, is easiest to control in a head-fixed animal. For this reason, under anaesthesia, we will attach a head-fixation device directly to the animal's skull and perform cranial surgery if necessary. The aim of this procedure is to allow painless and strong head-fixation of an anesthetized or awake animal and to allow insertion of probes or allow optical recording. This procedure can involve a combination of the following sub-procedures:

- Attachment of head fixation post
- Making a cranial window for optical recordings
- Making the skull transparent for optical recordings
- Making a cranial chamber for probe recordings
- Permanent insertion of a fibre, cannula or probe into the brain.

This procedure may take place before the visual deprivation and experimental manipulation, or at the start of the acute in vivo readout session.

2. Plasticity paradigm – Visual deprivation

We study the mechanisms of plasticity in the visual system. With this aim, we use the following common manipulations to induce plasticity by visual deprivation:
2a. Monocular deprivation, by eyelid suture or intraocular injection.
2b. Retinal lesion, by laser photocoagulation, injections or enucleation.

3. Experimental manipulation

We will perturb the brain by modulating signal processing using a variety of non-physiological means. The specific aim of these manipulation is one or more of the following

- To study the role of a class of neurons or area of the brain
- To study the role of a gene, protein or molecule
- To alter brain development and function

For modulating neural processing, we will use one or more of the following techniques
3a. Focal modulation
3b. Activation of designer proteins
3c. (Opto)pharmacology
3d. Non-standard housing conditions

4. Readout – Acute

The readout in these animals can involve measurements in a single acute in vivo experiment and a series of ex vivo analyses. These consist of a combination of the following procedures:
4a. Acute in vivo probe recording, measurements in awake or anaesthetised mice using a recording probe, e.g. an extracellular electrode, an intracellular pipette, or an electrochemical probe.
4b. Acute in vivo imaging, optical measurements in awake or anaesthetised mice of brain structure and activity. Examples are imaging of GFP or genetically coded calcium indicators in a selection of neurons, or wide-field intrinsic signal imaging.
4c. Slice physiology, where after euthanizing the mouse, its brain is sliced and used for electrophysiological and optical measurements of cellular activity and connectivity.
4d. Molecular analysis, where after euthanizing, the brain is processed to measure the molecular content in specific brain areas or cell types.
4e. Histology, where after euthanizing, the brain is sliced and possibly stained using immunohistochemistry or in situ hybridization and subsequently imaged.

The primary outcome of the experiments is the role of specific classes of neurons, brain areas, molecular, genes or proteins in visual processing. The primary outcome parameters for the recording and imaging experiments are the neuronal spiking or synaptic activity, or measurements of field potential or neurotransmitter release of specific groups of neurons. For the molecular analysis, this will be mainly the expression levels of specific genes or proteins in defined brain areas and specific neuronal groups. For the histology, this will be expression levels and connectivity of specific brain areas and neuronal populations. The histology may also be used to determine the success of the experimental manipulation, especially during the phase of the experiments that we are introducing and optimizing a (novel) technique.

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

1. Instrumentalization

We need to instrumentalize the mouse by using genetic manipulation and cranial surgery to make it possible to record from the brain or to perturb its function. Using genetic manipulation, we will express different classes of proteins:
Fluorescent proteins. Among these are proteins that constitutively fluorescent like the XFP family (such as eGFP, tdTomato and others), activity dependent fluorescent proteins (like GCaMPx and voltage-sensitive fluorescent proteins) and proteins that fluoresce if specific signal-cascades are activated (like Glu-sniffer or cAMP-dependent fluorescent proteins).
Designer signalling proteins to manipulate the responses and signalling in cells. Among these are proteins that can be activated by light (optogenetic proteins) or other means (such as sound) to allow ion flow (like ChR2 and variants), produce ion transport (like halorhodopsin, Arch, Shark and variants) or initiate a signalling cascade. A second subgroup are designer proteins, which allow ion flow, produce ion transport or initiate signalling cascade when activated by a ligand, such as CNO.
Variants of endogenous signalling proteins. We will also study the role of specific naturally occurring proteins in sensorimotor transformations. To study these proteins, we will overexpress the proteins themselves or altered versions of them (like constitutively active variants or dominant negative version). Among the proteins that we study in this way or BDNF, NF1 and GABA receptors.

The expression of these proteins will be done by three procedures listed below.

The percentage behind all the procedures is our estimate of the fraction of mice described in this appendix that will undergo the particular procedure. In these percentages we leave the in utero electroporation mothers out of the consideration. In the calculation of the number of mice, we do include these mothers.

Procedure 1a. Mouse lines. (instrumentalization for 100 % of the mice)
We will use wild type, spontaneous mutant and inbred mice and transgenic and knock-out/knock-in mouse lines.

Wild type, spontaneous mutant and inbred lines. For many experiments we can use standard inbred (or outbred) lines, such as C57Bl/6 which are commonly used for mouse vision and plasticity research. For some experiments, it is beneficial to use different inbred lines, such as CBA for in utero electroporation. In addition, we use inbred lines which have a naturally occurring mutation with an effect on plasticity in the visual system, such as ocular albino strains or slow-Wallerian degeneration mice; these lines have no affected phenotype causing discomfort.

Transgenic and knock-out/knock-in mice. In many cases we make use of genetically modified mouse lines. These mouse lines may express proteins to assess neuronal morphology or –function, designer proteins to alter neuronal function, or mutant forms of endogenous proteins. Cell-type- or brain-region-specific expression and temporal control is achieved by the use of appropriate promoters driving these genes, or making use of conditional gene expression systems. This may involve inducible promoters such as the tetO/tTA system or DNA-recombinases such as cre- or dre-recombinases or flippase. For conditional mutant mice, interbreeding of two or more mouse lines is required in order to achieve the required gene modification in the appropriate cell type or brain region.

Procedure 1b. In utero electroporation. (instrumentalization for 40% of the mice)
To express fluorescent proteins, designer signalling proteins or variants of endogenous signalling proteins we will use in utero electroporation where DNA or virus is injected into one of the brain ventricles of an

embryo and voltage pulses are used to transfect neurons and glial cells lining the ventricles.

Pregnant mice around two weeks of gestation (dependent on the targeted area or cell type) are anaesthetised. A midline abdominal incision is made to expose the uterine horns. A solution of the encoding plasmid and a colouring agent (like Fast Green) is injected in of the brain ventricles of the embryos through a capillary pipette. Electroporation is done with a series of pulses with a pulse generator. After electroporation the uterine horns are placed back in the abdominal cavity and abdomen is sutured, and the animal recovers from surgery. During the surgery care embryos are kept moist with warm saline, and handling is kept to a minimum. Animals are kept on a euthermic pad during the surgery, and receive postoperative analgesia.

Procedure 1c. Viral expression. (instrumentalization for 50% of the mice: all ages)
To express fluorescent proteins, designer signalling proteins or variants of endogenous signalling proteins we will use injections of different viruses, which each have specific advantages:
Viral vectors to obtain broad expression, such as AAV vectors. Serotypes will include AAV2/1, AAV2/5 and AAV2/9 and possibly others depending on the targeted area and cell classes. Viral transfections depends on the combination of serotype, area, and cell classes and are made into the brain or eye.
Retroviral vectors when integration of the plasmid is necessary for lineage studies, such as lentiviruses.
Retrograde viral vectors for connectivity studies, such as CAV viruses and some AAV serotypes.
Transsynaptic viral vectors for cell-specific connectivity studies, such as EnvA-pseudotyped rabies viruses. To comply with GGO rules it may be necessary to obtain a blood sample from a viral injected animal to assess the viral load in the circulation; maximally 100 ul from tail of facial vein.

For viral injections, mice are anesthetized. Pups (P0-P5) are sedated by hypothermia. For intracranial injections, one or more small craniotomies are made to allow insertion of a micropipette to the targeted brain area. Using a micro-volume injector small amounts of a solution containing the viral particles are inserted (less than 1 microliter) into the brain or eye. After the injections, the skin is sutured if necessary and the animal is allowed to recover. Analgesia is given if necessary. The virus is allowed to express for several days or weeks depending on the type. For the majority of the experiments, an injection session suffices, but for transsynaptic tracing such as those involving EnvA-pseudotyped rabies virus and a helper virus, two sessions are necessary.

Procedure 1d. Cranial surgery. (instrumentalization for 80% of the mice: all ages)
For recording neural activity and for delivering reproducible stimuli, it is often necessary to head fix the animal. For optical recording it is necessary to remove the skin and place a cranial (glass) window or make the skull transparent. For repeated electrical recordings it may be necessary to make a cranial chamber. For later manipulation or recording brain activity, it may be necessary to implant a fibre or probe into the brain and attach it to the skull. The surgical procedures below all require surgical anaesthesia and can be combined into a single surgical session for the majority of mice, and will maximally be done in two sessions. The entire surgery takes between 15 minutes and 4 hours.
Surgical procedure. The mouse is anesthetized and placed in a stereotact. The scalp is incised and possibly partially removed. After one or more of the procedures below, the skin is sutured, if necessary, for juvenile and mature animals. For pups, natural healing is expected to occur unassisted. Next, the animal allowed to recover. Analgesia is given if necessary. The animal is on a feedback-controlled heating pad during the procedure and recovers in a warm environment.
Attachment of head fixation post. Part of the scalp is removed and a metal handle is attached to the skull by glue and/or dental cement to allow later fixation to the setup.
For all experiments where mice will later be head-fixed while awake, the surgery is followed by a period of handling the animal and letting it become accustomed to being head-fixed.
Making a cranial window for optical recordings. First a small craniotomy is made. For imaging of individual cells and subcellular structures, the craniotomy is covered by a glass coverslip, permanently secured by glue and/or dental cement.
Making the skull transparent for optical recordings. The skull is covered by a layer of acrylic glue and polishing factor (such as nail polish). After drying, the skull is polished. This allows wide-field imaging of neural populations.
Making a cranial chamber for probe recordings. A small part of the scalp is removed. A small wall of dental cement is applied surrounding a small craniotomy. The craniotomy is covered with a removable substance (like silicon or bone wax) and which is possibly covered by another, but also removable, layer of harder material (like dental cement or epoxy). For added stability, miniscrews could be screwed into the skull after drilling to anchor the head fixation post. These screws may also be used as ground or reference contact points for electrophysiology.

Insertion of a probe, cannula, fibre or imaging accessories into the brain. A small craniotomy is made and a (electrode) probe, cannula, fibre or imaging accessories (such as a GRIN lens or microprism) or is inserted into the brain using a micromanipulator. The top of the probe, cannula, fibre or imaging accessory is then attached to the skull, and possibly covered.

2. Plasticity paradigm – Visual deprivation

For inducing plasticity by visual deprivation, we use two procedures. We have experience with both procedures and there is already an extensive literature using both procedures.

Procedure 2a. Monocular deprivation. (procedure for 95% of the mice)
The mouse is anesthetized and its temperature is maintained. One eyelid is sutured closed. Alternatively, an inactivating drug may be injected intraocularly to obtain silencing of retinal responses. After the procedure, the mouse recovers from anaesthesia. Perioperative analgesia is applied if necessary. The surgery takes about 30 minutes. The lid suture procedure may be followed by a later session under anaesthesia to reopen the sutured lid. In a minority of the experiments, this could be followed by another session of monocular deprivation of the same or the other eye.

Procedure 2b. Retinal lesion. (procedure for 5% of the mice)
The mouse is anesthetized and its temperature is maintained. The mouse is placed under a microscope. Monocular or binocular retinal lesions are made by laser photocoagulation or drug injection. Alternatively, one or both eyes are enucleated. For enucleation, post-surgical analgesia is given. After the procedure, the mouse recovers from anaesthesia. This procedure happens only once per animal. The surgery takes about 15 minutes.

3. Experimental manipulation

We may perturb the brain acutely or chronically to manipulate information processing and plasticity, using the following methods, mostly applied alone but also in combination (estimated less than 10%).

Procedure 3a. Focal modulation (experimental manipulation for 10% of the mice)
We will locally stimulate or inhibit neural activity using transcranial, local and intracellular electrical stimulation and inactivation, and focal ultrasound delivery. Focal modulation is typically applied for short intervals in the order of seconds, but these intervals can be repeated for several minutes. Typically focal modulation is done during readout, and will have a similar frequency.

Procedure 3b. Activation of designer proteins. (experimental manipulation for 30% of the mice)
Following the expression of designer proteins, we will activate them. This can be using non-invasive means such as the applying light (for optogenetic proteins) through the air or through a fibre, or sound (for mechanogenetic proteins) onto the skull, or by injecting or supplying the ligand (such as CNO in the case of DREADDs) to the animal. The ligand delivery can be by food or liquid supplements, systemic i.p. injections, local delivery through a cannula in the awake animal, or local delivery during the readout session. The effect of activation will depend on the specific designer protein expressed, but could be a change in the membrane polarization or the (de)activation of an intracellular signalling cascade. The activation should have little side effects apart from activating the designer proteins. Light or sound are typically applied for short intervals in the order of seconds, but these intervals can be repeated for several minutes. The application is usually done during readout, and will have a similar frequency. Supplying a ligand using food or liquid supplements, systemically or via a cannula will usually be only during one single period in the experiment, but this period can last a number of days. We will choose the administration route that causes the least discomfort while providing a delivery of a sufficient and consistent dose.

Procedure 3c. (Opto)pharmacology. (experimental manipulation for 20% of the mice)
We will administer or focally apply drugs and control substances. The drugs will target the nervous system and modulate synaptic transmission (for instance NMDA-receptor antagonists and GABAR agonists), neuromodulation (e.g. cholinergic, serotonergic, dopaminergic) or other neural functions, but also include silencers and excitotoxins (like ibotenic acid). Furthermore, we use drugs preventing or inducing the expression of genes (inserted through procedure 1) such as doxycycline or tamoxifen. The drugs could also be photoactivatable, where the extent of activation will be controlled by light-activation. Control substances could be regular saline, or saline with the same solvent, or non-active compound that is used for the drug delivery. Administration routes will generally be one or more intraperitoneal injections, food or liquid supplements, or local delivery through a cannula in awake animals or local delivery during readout. Supplying a ligand using food or liquid supplements, systemically or via a

cannula will usually be only during one single period in the experiment, but this period can last a number of days. We will choose the administration route that causes the least discomfort while providing a delivery of a sufficient and consistent dose.

Procedure 3d. Non-standard housing conditions. (experimental manipulation for 20% of the mice)
Housing can have an influence on brain development and function. Commonly we will use standard housing conditions, but we will also use non-standard housing conditions. In particular, we will use three common procedures, with which we have previous experience.
Dark rearing. Pregnant mothers are placed in a completely dark environment. Pups are born and raised in this dark environment. Cage changing and food and water delivery will be done in dim red-light conditions, which should be not visible to mice. Dark rearing has been shown to delay maturation of the visual system.
Dark housing. Animals are housed in a completely dark environment for several days. Cage changing and food and water delivery will be done in dim red-light conditions, which should be not visible to mice. Dark housing has been shown to enhance plasticity in the visual system.
Environmental enrichment. Animals are housed in large enriched environment providing ladders, boxes, wheels and other accessories. Environmental enrichment has previously been shown to have a positive effect on visual plasticity.

4. Readout – Acute

To determine the effects of visual deprivation, we make a number of physiological measurements using common procedures for which we have much experience. The measurements can be combined, and therefore the percentages for frequency of the procedures add up to above 100%.

Procedure 4a. Acute in vivo probe recording. (readout for 50% of the mice)
In a single head-fixed session, directly followed by euthanasia, we will record brain activity using an invasive probe, such as an extracellular microelectrode, intracellular pipet or electrochemical probe. The recording session can start with induction of anaesthesia, but may also be in an awake animal. For most awake experiments, the cranial surgery will have taken place multiple days or weeks before this recording session. For experiments, that only require recording under anaesthesia, the cranial surgery as described in procedure 1d may be performed at the start of this acute session. The recording probe may already be in place at the start of the session or will otherwise be inserted through the craniotomy that was made as described in procedure 1d.

Procedure 4b. Acute in vivo imaging. (readout for 50% of the mice)
In a single head-fixed session (possibly combined with procedure 4a), directly followed by euthanasia, we will use a microscope or a macroscope (low magnification imaging setup) to image structure and activity of the brain using, for instance, intrinsic signal, flavoprotein, fluorescent dyes or fluorescent proteins. The imaging session can start with induction of anaesthesia, but may also be in an awake animal. For most awake experiments, the cranial surgery will have taken place multiple days or weeks before this recording session. For experiments that only require recording under anaesthesia, the cranial surgery as described in procedure 1d may be performed at the start of this acute session. We will use the cover slip or other imaging accessories (such as GRIN lens or microprism) that have been placed during the instrumentalization procedure of the animal. To image deeper than a few hundred microns, we may also remove part of the overlying brain structures in experiments under anaesthesia.

Procedure 4c. Slice physiology. (readout for 10% of the mice)
Mice are anesthetized (possibly following procedures 4a-b) and euthanized by decapitation. The brains will then be dissected and sliced for acute slice physiology experiments. In the slices we will measure activity at the network, cellular and synaptic levels using electrophysiology and imaging.

Procedure 4d. Molecular analysis. (readout for 10% of the mice)
Mice are anesthetized (possibly following procedures 4a-b) and euthanized by decapitation. The brains will then be dissected and processed to determine protein, DNA, RNA or biochemical content.

Procedure 4e. Histology. (readout for 50% of the mice)
The animal will euthanized with an overdose of anaesthetic and when it is sufficiently deeply anesthetized, it will be transcardially perfused with PBS and fixative (such as 4% PFA in PBS). After the perfusion the brain is removed from the skull and kept for a period to enhance fixation. Typically, the brain is then sliced and stained using standard immunohistochemistry protocols. The stained slices are then mounted on microscope slides and imaged using a microscope. We will also use tissue clearing

methods on whole brains or large tissue sections, before staining.

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

Pilot experiments
Statistical methods do not apply. Establishing new manipulations requires testing and adaptation based on obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Initial 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 and origin
We will use mice (Mus musculus) in these experiments. This choice is based on the amount of fundamental knowledge that exists about the anatomy and physiology of the mouse brain and the availability of sophisticated technologies for investigating brain mechanisms, including a variety of genetically engineered strains. We include different mouse lines, including wild-type (e.g. C57Bl6) and genetically modified animals. The choice of transgenic animals depends on the specific research question. For example, if we need to measure activity of specific neurons, we can make use of transgenic mouse line that expresses, for example, a calcium indicator in a specific subclass of interneurons. We will carefully consider the best mouse model for every research question separately, taking latest developments into account. All mice are derived from the NIN, an establishment licensed by the NVWA, or from a registered commercial company.
In the animals in which variants of endogenous proteins are expressed or knocked-out it may be possible that there is discomfort as a result of an affected phenotype, but no discomfort is reported for any of the lines that we intend to use. For this protocol we assume that there is no congenic discomfort for the breeding of any of the lines. New lines will be monitored for two generations and the outcome will be presented to the IvD. In the unlikely event we want to use a strain with congenital discomfort we will add an addendum for such a mouse line to the CCD protocol.

Sex
For readout, we will use both males and females from the animal lines that are bred in-house, to reduce the number of surplus animals. For most of our questions in our line of research (plasticity in the visual system) we do not expect any differences between genders. However, we will document the gender and investigate if there are any gender differences that are relevant for our research questions. If so, we will ask for approval of the IVD to only use mice of the appropriate sex for such experiments.

Life stages
We will only do experiments involving visual stimulation in animals after eye opening. Mice open their eyes not before postnatal day P10, but manipulations may need to be done earlier, e.g. transfection takes several days after viral injection. The majority of mice will consist of adults and juveniles after weening at P21. In mice, vision deteriorates with age, so most experiments will be in mice that are less than 1 year old. In utero electroporation will take place in embryos not younger than E10. Mothers used for in utero electroporation will be adult.

Animal number
The estimate of the total number of experimental groups is primarily based on our experience over the past years performing very similar experiments. There are about a dozen researchers in our laboratories.

We expect them together to do about 5 separate experiments (e.g. the role of a specific gene in plasticity in a visual area) with this procedure per year. Based on previous experiments over the last decade, we estimate that we need about 50 animals for the readout per experiment. This would amount to 50 * 5 * 5 (animals/experiment * experiments/year * years) = **1250 animals**. We estimate that 1 out of 5 experiment use in utero electroporation, i.e. 250 animals. Based on previous experience, we obtain about 3 usable electroporated pups per mother. We would thus require 250 / 3 = **83 mothers**. The requested number of animals is thus 1250 + 83 = **total 1333**.

When possible, we will at the start of an experiment carefully determine the number of animals needed with a power-analysis, or we will start with a pilot-experiment. We will stop the experiments once the aims of the experiment have been achieved and not use further mice.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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

☒ No

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

D. Replacement, reduction, refinement

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

Replacement
To disentangle the brain mechanisms that underlie plasticity in the mammalian visual system, we cannot use cell cultures or animal species with a very different visual system such as other vertebrates that lack a cerebral cortex. Intact brains of awake animals need to be studied to understand how brain activity underlies visual perception and learning. We can only study the cellular level and, in particular, the contribution of specific cells types (e.g. the interneurons) and changes in the connectivity that result from plasticity in mice due to the powerful methods that exist in this animal model (e.g. calcium imaging, 2-photon imaging, optogenetics, transgenic animals). The possibilities for invasive measurements in the human brain are limited. Furthermore, there are no models available with enough level of accurate detail to answer the same questions. Hence, the replacement of the mice needed for these experiments, is not possible.

Reduction
We reduce the number of animals to the minimum possible. First, we will calculate the number of animals needed to achieve a certain statistical power beforehand, in case we know the effect size. If the effect size is unknown, we will first do a pilot experiment using only a few animals to determine the expected effect size. Second, we will use both male and female mice whenever possible to reduce the number of surplus animals. Third, whenever possible we use animals for both in vivo and ex vivo studies.

Refinement
We use a collection of the newest genetic techniques to make our experimental manipulations as specific as possible. Furthermore, all surgeries will be carried out by persons who are well trained. Animals are housed in cages with cage enrichment in order to keep them engaged which we believe to reduce discomfort of living in a cage and improve cognitive capabilities.

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

Animals will be housed socially, unless it is impossible because of the risk of damage to a specific cranial implant or eye suture. Our experience shows that mice with simple cranial windows and head posts can continue to be housed socially.

During surgeries, analgesics and anaesthesia are used to minimize pain and suffering. Breathing and temperature will be registered and level of anaesthesia and warmth of heating-pad will be adjusted as such. Peri-surgical analgesics will be administered if necessary and animals will wake up in a warm environment. Care will be taken to facilitate easy access to food and water. Mice will be allowed to recover for at least two days following surgery. Behaviour, wound area, weight and appearance will be monitored daily at least for 3 days post-surgery and longer for surgeries that would have a longer chance of complications.

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.

For some cranial implants, it may be necessary to house mice solitarily after a surgery or brain injection to prevent cage mates damaging surgical wounds or the 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. Mice cage will be enriched by bedding material and also running wheels, pipes and/or shelters when possible.

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.

During surgery we will use adequate anaesthesia and analgesia. Also post-surgery analgesics will be administered. Furthermore, before killing any mice, they will be deeply anesthetized.

I. Other aspects compromising the welfare of the animals

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

Infections.
In rare cases there is a possibility of infection around the wound area. In these cases, we will apply additional analgesics and/or antibiotics. Visible signs of pathogenesis will be monitored. The following will be considered as signs of an unhealthy state of the animal: aberrant behaviour, dehydration, weight loss, nose and mouth discharge.

Insufficient recovery after surgery.
Applicable if an animal shows weight loss (more than 15% of its post-surgery weight within two days, or more than 20% in total). This occurs infrequently (<2%).

Head post detachment.
If the head post breaks off, the animal is immediately anesthetized to allow assessment of the discomfort level. If the head post becomes loose/detached due to bad adhesion to the skull we are able to reattach the head post in an immediate repair surgery. The repair surgery is performed under anaesthesia and with analgesia in an identical fashion to the original attachment surgery, with the exception that the skin does not need to be cut, this makes the repair surgery both shorter in duration and less invasive than the original implantation surgery. We estimate the discomfort to be moderate during recovery from the anaesthesia (1 day) becoming mild for 1-2 days. In the rare case (<2%) that the detachment of the head post damages the skull, the mouse is immediately euthanized under anaesthesia.

Reopening of craniotomy.
In rare cases (<5%), the craniotomy is reopened causing moderate discomfort. In these cases the mouse is immediately euthanized under anaesthesia.

Explain why these effects may emerge.

Mild to moderate discomfort due to unintended effects as described above can occur in a small percentage of mice. The head post can become loose due to the forces exerted by the mouse on the head post while fixed in the set-up.

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

We monitor the animal's behaviour, wound area and physiology in these days after surgery. The surgeries are performed using adequate surgical procedures in semi-sterile conditions and without unnecessary delays to minimize the amount of time the animal is under anaesthesia. Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD and/or veterinary officer. If necessary, treatment will be initiated (topically or systemically applied medication).

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.

Each animal that undergoes surgery will be monitored for clinical parameters. The mice will be monitored for their general appearance and activity level. If a mouse has an appearance that gives cause for concern (e.g. signs of infection around the wound, hair standing up or sunken flanks, reduction in activity level) then we will notify the IvD. Similarly, if the animal does not recover well from anesthesia we will evaluate the animal together with the IvD.
In addition, the weight of the animal will be monitored and if the animal loses 15% of their weight in two consecutive days or if the animal loses more than 20% of their weight throughout the course of the experiment then the IvD will be contacted and a decision will be made whether a humane endpoint has been reached. If the headpost breaks off damages the skull or reopening of the craniotomy occurs, the mouse will be immediately euthanized.

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').

The cumulative discomfort for all mice, including mothers, in this appendix is **moderate**.

The discomfort levels of individual procedures is as follows:

1a. Transgenic mouse lines.
No discomfort is expected.

1b. In utero electroporation.
Moderate discomfort from surgery is expected for all mothers. Mild discomfort is expected for the embryos.

1c. Viral expression.
Moderate discomfort (for 1-2 days) becoming mild (for 2-3 days) is expected for intracranial viral injections. The procedure is combined with 1d whenever possible.

1d. Cranial surgery.
Moderate discomfort (for 1-2 days) becoming mild (for 2-3 days) is expected for intracranial viral injections. The procedure is combined with 1c whenever possible.

2a. Monocular deprivation, by eyelid suture or intraocular injection.
Moderate discomfort (for 1 day) from surgery.

2b. Retinal lesion, by laser photocoagulation or enucleation.
Mild or maximally moderate discomfort (for 1 day) from surgery.

3a. Focal modulation.
No discomfort when applied during anesthetized readout or after slicing.
Mild discomfort when applied to awake animal, due to disoriented feeling due to focal brain modulation.

3b. Activation of designer proteins.
No discomfort when applied during anesthetized readout or after slicing.
Mild discomfort when applied to awake animal, due to disoriented feeling due to brain activation, or stress due to drug delivery.

3c. (Opto)pharmacology
No discomfort when applied during anesthetized readout or after slicing.
Mild discomfort when applied to awake animal, due to disoriented feeling due to brain activation, or stress due to drug delivery.

3d. Non-standard housing condition
Mild discomfort due to change of housing conditions for enrichment.
Moderate discomfort in dark housing is possible because of lack of vision and disturbed circadian rhythm.

4a. Acute in vivo probe recording
Mild discomfort due to induction of anaesthesia for recording under anaesthesia.
Moderate discomfort due to head fixation in awake recording.

4b. Acute in vivo imaging
Mild discomfort due to induction of anaesthesia for imaging under anaesthesia.
Moderate discomfort due to head fixation in awake imaging.

4c. Slice physiology
Mild discomfort due to induction of anaesthesia if the mouse was not already anaesthetized for 4a or 4b.

4d. Molecular analysis

Mild discomfort due to induction of anaesthesia if the mouse was not already anaesthetized for any of procedure 4a-c.

4e. Histology
Mild discomfort due to induction of anaesthesia if the mouse was not already anaesthetized for any of procedure 4a-c.

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.

For ex vivo histology or molecular analysis, or for slice physiology it is necessary to kill the animal and extract the brain.

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'.	80100-KNAW	
1.2	Provide the name of the licenced establishment.	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 **Readout during visual deprivation:** Chronic in vivo assessment of neuronal function or anatomy in control mice and mice during plasticity induction by visual deprivation.

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 aim of this procedure is to measure the effect on neural structure and function by an experimental manipulation in mice during and after plasticity induced by visual deprivation.

The general design has the following components:
1. Instrumentalization of the experimental animal
2. Plasticity paradigm - visual deprivation
3. Experimental manipulation
4. Readout – chronic and terminal

These components are described in detail in Appendix 3.4.4.1, and are not repeated here.

Added for this appendix are the chronic readout components:

4. Readout – Chronic

We want to observe the changes in the brain while plasticity takes places. For this reason we would like to record and image the structure and functioning of visual system during plasticity. To obtain a baseline of the dynamics will we commence measuring before plasticity induction and continue measuring after the period of visual plasticity. Chronic readout consist of one or both of the following procedures:
4f. Chronic in vivo probe recording, repeated measurements in awake or anaesthetised mice using a recording probe, e.g. an extracellular electrode, an intracellular pipet, or an electrochemical probe taking

place over multiple sessions.
4g. Chronic in vivo imaging, repeated optical measurements in awake or anaesthetised mice of brain structure and activity over multiple sessions. Examples are imaging of GFP in a selection of neurons, genetically coded calcium indicators, or wide-field intrinsic signal imaging.

The primary outcome parameters of these chronic recording and imaging experiments are the dynamics in the changes in neuronal structure and spiking or synaptic activity of specific groups of neurons induced by the visual plasticity induction paradigm while manipulation the brain.

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

The procedures are described in detail in Appendix 3.4.4.1, and are not repeated here.
Two readout procedures are added:

For all experiments, the cranial surgery will have taken place multiple days or weeks before the first recording and imaging session. Typically, we will record or image for less than 10 sessions. In some cases, however, it may be that plasticity continues to change the visual system. In these cases, we may record more sessions, but we do not expect that ever more than 20 sessions will be useful. Typical experiments would have no more than 1 session per day. If, however, changes occur during a day but take longer than a single session could last, it may be necessary in exceptional cases to measure during multiple sessions on a single day. We will discuss the necessity of this with the IvD.

Procedure 4f. Chronic in vivo probe recording (readout for 75% of the mice)
In multiple sessions, we will record brain activity using an invasive probe, such as an extracellular microelectrode, intracellular pipet or electrochemical probe. The recording session can start with induction of anaesthesia, but in the majority of cases will be in an awake animal. Awake recordings may be necessary to witness the brain during normal function. Under anaesthesia, information processing and plasticity mechanisms are reduced or altered. Awake recording can be done in head-fixed animals or freely moving animals using a tethered or wireless connection. For head-fixed awake sessions, mice will have been habituated to being head-fixed as part of the cranial surgery procedure 1d.

Procedure 4g. Chronic in vivo imaging. (readout for 75% of the mice)
In multiple sessions (possibly combined with procedure 4f), we will a microscope or a macroscope (low magnification imaging setup) to image structure and activity of the brain using, for instance, intrinsic signal, flavoprotein, fluorescent dyes or fluorescent proteins. The imaging session can start with induction of anaesthesia, especially for structural imaging, but can also be in an awake animal, especially for functional imaging. Under anaesthesia, information processing and plasticity mechanisms are reduced or altered. Awake imaging can be done in head-fixed animals or freely moving animals using optical fibres or miniature microscopes. For head-fixed awake sessions, mice will have been habituated to being head-fixed as part of the cranial surgery procedure 1d.

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

(Text in black is identical to Appendix 3.4.4.1. In red are the differences.)

Pilot experiments
Statistical methods do not apply. Establishing new manipulations requires testing and adaptation based on obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Initial 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. By taking multiple longitudinal measurements in the same mice, we can do paired statistics which will have more power than comparing

visually deprived mice with other non-deprived control animals.

B. The animals

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

(Text in black is identical to Appendix 3.4.4.1. In red are the differences.)

Species and origin

We will use mice (Mus musculus) in these experiments. This choice is based on the amount of fundamental knowledge that exists about the anatomy and physiology of the mouse brain and the availability of sophisticated technologies for investigating brain mechanisms, including a variety of genetically engineered strains. We include different mouse lines, including wild-type (e.g. C57Bl6) and genetically modified animals. The choice of transgenic animals depends on the specific research question. For example, if we need to measure activity of specific neurons, we can make use of transgenic mouse line that expresses, for example, a calcium indicator in a specific subclass of interneurons. We will carefully consider the best mouse model for every research question separately, taking latest developments into account. All mice are derived from the NIN, an establishment licensed by the NVWA, or from a registered commercial company.

In the animals in which variants of endogenous proteins are expressed or knocked-out it may be possible that there is discomfort as a result of an affected phenotype, but no discomfort is reported for any of the lines that we intend to use. For this protocol we assume that there is no congenic discomfort for the breeding of any of the lines. New lines will be monitored for two generations and the outcome will be presented to the IvD. In the unlikely event we want to use a strain with congenital discomfort we will add an addendum for such a mouse line to the CCD protocol.

Sex

For readout, we will use both males and females from the animal lines that are bred in-house, to reduce the number of surplus animals. For most of our questions in our line of research (plasticity in the visual system) we do not expect any differences between genders. However, we will document the gender and investigate if there are any gender differences that are relevant for our research questions. If so, we will ask for approval of the IVD to only use mice of the appropriate sex for such experiments.

Life stages

We will only do experiments involving visual stimulation in animals after eye opening. Mice open their eyes not before postnatal day P10, but manipulations may need to be done earlier, e.g. transfection takes several days after viral injection. The majority of mice will consist of adults and juveniles after weening at P21. In mice, vision deteriorates with age, so most experiments will be in mice that are less than 1 year old. In utero electroporation will take place in embryos not younger than E10. Mothers used for in utero electroporation will be adult.

Animal number

The estimate of the total number of experimental groups is primarily based on our experience over the past years performing very similar experiments. There are about a dozen researchers in our laboratories. We expect them together to do about 5 separate experiments (e.g. the role of a specific gene in plasticity in a visual area) with this procedure per year. Based on previous experiments over the last decade, we estimate that we need about 30 animals for the readout per experiment. This is less than for a typical experiment where we only take a terminal measurement, because the longitudinal measurements have more power in a significance calculation. This would amount to 30 * 5 * 5 (animals/experiment * experiments/year * years) = **750 animals**. We estimate that 1 out of 5 experiments use in utero electroporation, i.e. 150 animals. Based on previous experience, we obtain about 3 usable electroporated pups per mother. We would thus require 150 / 3 = **50 mothers**. The requested number of animals is thus 750 + 50 = **total 800**.

At the start of an experiment, we will carefully determine the number of animals needed with a power-analysis, or we will start with a pilot-experiment. We will stop the experiments once the aims of the experiment have been achieved and not use further mice.

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.

(Text in black is identical to Appendix 3.4.4.1. In red are the differences.)

Replacement
To disentangle the brain mechanisms that underlie plasticity in the mammalian visual system, we cannot use cell cultures or animal species with a very different visual system such as other vertebrates that lack a cerebral cortex. Intact brains of awake animals need to be studied to understand how brain activity underlies visual perception and learning. We can only study the cellular level and, in particular, the contribution of specific cells types (e.g. the interneurons) and changes in the connectivity that result from plasticity in mice due to the powerful methods that exist in this animal model (e.g. calcium imaging, 2-photon imaging, optogenetics, transgenic animals). The possibilities for invasive measurements in the human brain are limited. Furthermore, there are no models available with enough level of accurate detail to answer the same questions. Hence, the replacement of the mice needed for these experiments, is not possible.

Reduction
We reduce the number of animals to the minimum possible. First, we will calculate the number of animals needed to achieve a certain statistical power beforehand, in case we know the effect size. If the effect size is unknown, we will first do a pilot experiment using only a few animals to determine the expected effect size. Second, we will use both male and female mice whenever possible to reduce the number of surplus animals. Third, whenever possible we use animals for both in vivo and ex vivo studies. Fourth, by taking repeated longitudinal measurements in one animal, we reduce the number of animals needed.

Refinement
We use a collection of the newest genetic techniques to make our experimental manipulations as specific as possible. Furthermore, all surgeries will be carried out by persons who are well trained. Animals are housed in cages with cage enrichment in order to keep them engaged which we believe to reduce discomfort of living in a cage and improve cognitive capabilities.

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

(Text is identical to Appendix 3.4.4.1.)

Animals will be housed socially, unless it is impossible because of the risk of damage to a specific cranial implant or eye suture. Our experience shows that mice with simple cranial windows and head posts can continue to be housed socially.

During surgeries, analgesics and anaesthesia are used to minimize pain and suffering. Breathing and temperature will be registered and level of anaesthesia and warmth of heating-pad will be adjusted as such. Peri-surgical analgesics will be administered if necessary and animals will wake up in a warm

environment. Care will be taken to facilitate easy access to food and water. Mice will be allowed to recover for at least two days following surgery. Behaviour, wound area, weight and appearance will be monitored daily at least for 3 days post-surgery and longer for surgeries that would have a longer chance of complications.

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.

(Text is identical to Appendix 3.4.4.1.)
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.

(Text is identical to Appendix 3.4.4.1.)

For some cranial implants, it may be necessary to house mice solitarily after a surgery or brain injection to prevent cage mates damaging surgical wounds or the 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. Mice cage will be enriched by bedding material and also running wheels, pipes and/or shelters when possible.

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.

(Text is identical to Appendix 3.4.4.1)
During surgery, we will use adequate anaesthesia and analgesia. Also post-surgery analgesics will be administered. Furthermore, before killing any mice, they will be deeply anesthetized.

I. Other aspects compromising the welfare of the animals

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

(Text is identical to Appendix 3.4.4.1.)

Infections.
In rare cases there is a possibility of infection around the wound area. In these cases, we will apply additional analgesics and/or antibiotics. Visible signs of pathogenesis will be monitored. The following will be considered as signs of an unhealthy state of the animal: aberrant behaviour, dehydration, weight loss, nose and mouth discharge.

Insufficient recovery after surgery.
Applicable if an animal shows weight loss (more than 15% of its post-surgery weight within two days, or more than 20% in total). This occurs infrequently (<2%).

Head post detachment.
If the head post breaks off, the animal is immediately anesthetized to allow assessment of the discomfort level. If the head post becomes loose/detached due to bad adhesion to the skull we are able to reattach the head post in an immediate repair surgery. The repair surgery is performed under anaesthesia and with analgesia in an identical fashion to the original attachment surgery, with the exception that the skin does not need to be cut, this makes the repair surgery both shorter in duration and less invasive than the original implantation surgery. We estimate the discomfort to be moderate during recovery from the anaesthesia (1 day) becoming mild for 1-2 days. In the rare case (<2%) that the detachment of the head post damages the skull, the mouse is immediately euthanized under anaesthesia.

Reopening of craniotomy.
In rare cases (<5%), the craniotomy is reopened causing moderate discomfort. In these cases the mouse is immediately euthanized under anaesthesia.

Explain why these effects may emerge.

(Text is identical to Appendix 3.4.4.1.)

Mild to moderate discomfort due to unintended effects as described above can occur in a small percentage of mice. The head post can become loose due to the forces exerted by the mouse on the head post while fixed in the set-up.

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

(Text is identical to Appendix 3.4.4.1.)

We monitor the animal's behaviour, wound area and physiology in these days after surgery. The surgeries are performed using adequate surgical procedures in semi-sterile conditions and without unnecessary delays to minimize the amount of time the animal is under anaesthesia. Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD and/or veterinary officer. If necessary, treatment will be initiated (topically or systemically applied medication).

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.

(Text is identical to Appendix 3.4.4.1.)

Each animal that undergoes surgery will be monitored for clinical parameters. The mice will be monitored for their general appearance and activity level. If a mouse has an appearance that gives cause for concern (e.g. signs of infection around the wound, hair standing up or sunken flanks, reduction in activity level) then we will notify the IvD. Similarly, if the animal does not recover well from anesthesia we will evaluate the animal together with the IvD.
In addition, the weight of the animal will be monitored and if the animal loses 15% of their weight in two consecutive days or if the animal loses more than 20% of their weight throughout the course of the experiment then the IvD will be contacted and a decision will be made whether a humane endpoint has

been reached. If the headpost breaks off damages the skull or reopening of the craniotomy occurs, the mouse will be immediately euthanized.

Indicate the likely incidence.

(Text is identical to Appendix 3.4.4.1.)

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').

Cumulative discomfort for all animals, including the mothers, is classified as **moderate**

The classification of severity of the individual procedures is described in detail in Appendix 3.4.4.1, and is not repeated here. Two classifications are added below:

4f. Chronic in vivo probe recording
Moderate discomfort due to repeated recovery from anaesthesia for recording under anaesthesia.
Moderate discomfort due to head fixation in awake recording.

4g. Chronic in vivo imaging
Moderate discomfort due to repeated recovery from anaesthesia for recording under anaesthesia.
Moderate discomfort due to head fixation in awake imaging.

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.

As in Appendix 3.4.4.1.

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'.	80100-KNAW	
1.2	Provide the name of the licenced establishment.	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 3.4.4.3	Type of animal procedure **Readout after visual learning:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual learning

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 aim of this procedure is to measure the effect on neural structure and function by an experimental manipulation in mice after plasticity induced by visual learning.

The general design has the following components:
1. Instrumentalization of the experimental animal
2. Plasticity paradigm - visual learning
3. Experimental manipulation
4. Readout – acute

These components are described in detail in Appendix 3.4.4.1, and are not repeated here.
Added here are visual learning paradigms:

2. Plasticity paradigm – Visual learning

We study the mechanisms of plasticity in the visual system. With this aim, we use the following common manipulations to induce plasticity by visual learning:
2c. Perceptual learning, where visual stimuli are coupled to behavioural output by positive reinforcement training
2d. Fear learning, where visual stimuli and context are coupled to a fear response
2e. Unsupervised learning, where repeated visual stimuli change neural response and behaviour without reinforcement.

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

The procedures are described in detail in Appendix 3.4.4.1, and are not repeated here.
Three visual learning procedures are added:

2. Plasticity paradigm – Visual Learning

For inducing plasticity by visual learning, we will use the following procedures, with which we already have experience. In the majority of cases, plasticity is induced using only one of these procedures, but it may sometimes be useful to combine the procedures. Therefore, the percentages for frequency of the procedures add up to above 100%.

Procedure 2c. Perceptual learning. (visual learning for 50% of the mice)
During perceptual learning, the mouse is trained to associate specific visual stimuli with specific actions. Examples of stimuli could be gratings with a certain orientation, or pictures of objects. Examples of actions could by licking from a specific lick spout, button/lever push or turning a decision wheel/ball. The training is coupled with a positive reward when the mouse performs to right action for the presented stimulus. The positive reward will in an estimated 90% of the perceptual learning experiments of a liquid reward. Other possible rewards are food or sensory stimulus that has been associated to food or liquid reward. To enhance the value of a liquid reward, the animals will be held in a water control regime. On working days, they obtain water as a reward in performing the task correctly. On non-working days they receive a limited amount of water. The literature and previous experience (from another laboratory at the NIN and our own laboratory) suggests that this is better than providing ad libitum access on non-working days. When the mouse is under fluid control we monitor its appearance and behaviour every day. If signs of possible dehydration are observed (explained in detail in section I), the animal is temporarily removed from the trial and given ad libitum access to water. An individual training session on one day typically last 30 minutes to 2 hours, and can involve several hundred stimulus presentations. The whole learning can take up to 50 sessions, but we aim for perceptual learning paradigms that can be trained in less than 14 days. The performance of the mouse is monitored throughout the training period. The animals will be head-fixed in an estimated 90% of the perceptual learning experiments and freely moving in the remaining 10%.

Procedure 2d. Fear learning. (visual learning for 20% of the mice)
Animals will learn to fear a particular visual stimulus or context, by its association with a noxious stimulus. The noxious stimulus could be a light (foot / tail shock), strong sound, or an air puff. The animals will be head-fixed in an estimated 30% of the perceptual learning experiments. The fear learning typically is short period of several minutes in which the visual stimulus is paired to the noxious stimulus, and the response to the paired or another unpaired visual stimulus or context is measured during the same sessions or sessions in later days. The number of sessions in which a noxious stimulus is delivered is kept to a minimum. For the shock stimulus, we will never use more than five condition sessions.

Procedure 2e. Unsupervised learning (visual learning for 50% of the mice)
Seeing can also lead to changes in behaviour when it is not coupled to a reinforcement. Examples are the freezing and fleeing behaviour to bird-like stimuli flying overhead, and the approach behaviour to novel visual stimuli. We will present visual stimuli and observe behavioural response changing over multiple sessions.

For all the visual learning paradigms observe the mouse behaviour by video camera. For head-fixed sessions, we will also monitor the animal's physiology by pupil recordings (in most cases) and for some cases the heart rate and muscle activity.

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

(Text is identical to Appendix 3.4.4.1.)

Pilot experiments
Statistical methods do not apply. Establishing new manipulations requires testing and adaptation based on obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Initial 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.

(Text in black is identical to Appendix 3.4.4.1; in red are the differences)

Species and origin
We will use mice (Mus musculus) in these experiments. This choice is based on the amount of fundamental knowledge that exists about the anatomy and physiology of the mouse brain and the availability of sophisticated technologies for investigating brain mechanisms, including a variety of genetically engineered strains. We include different mouse lines, including wild-type (e.g. C57Bl6) and genetically modified animals. The choice of transgenic animals depends on the specific research question. For example, if we need to measure activity of specific neurons, we can make use of transgenic mouse line that expresses, for example, a calcium indicator in a specific subclass of interneurons. We will carefully consider the best mouse model for every research question separately, taking latest developments into account. All mice are derived from the NIN, an establishment licensed by the NVWA, or from a registered commercial company.
In the animals in which variants of endogenous proteins are expressed or knocked-out it may be possible that there is discomfort as a result of an affected phenotype, but no discomfort is reported for any of the lines that we intend to use. For this protocol we assume that there is no congenic discomfort for the breeding of any of the lines. New lines will be monitored for two generations and the outcome will be presented to the IvD. In the unlikely event we want to use a strain with congenital discomfort we will add an addendum for such a mouse line to the CCD protocol.

Sex
For readout, we will use both males and females from the animal lines that are bred in-house, to reduce the number of surplus animals. For most of our questions in our line of research (plasticity in the visual system) we do not expect any differences between genders. However, we will document the gender and investigate if there are any gender differences that are relevant for our research questions. If so, we will ask for approval of the IVD to only use mice of the appropriate sex for such experiments.

Life stages
We will only do experiments involving visual stimulation in animals after eye opening. Mice open their eyes not before postnatal day P10, but manipulations may need to be done earlier, e.g. transfection takes several days after viral injection. The majority of mice will consist of adults and juveniles after weening at P21. In mice, vision deteriorates with age, so most experiments will be in mice that are less than 1 year old. In utero electroporation will take place in embryos not younger than E10. Mothers used for in utero electroporation will be adult.

Animal number
The estimate of the total number of experimental groups is primarily based on our experience over the past years performing very similar experiments. There are about a dozen researchers in our laboratories. We expect them together to do about 5 separate experiments (e.g. the role of a specific gene in plasticity in a visual area) with this procedure per year. Based on previous experiments over the last decade, we estimate that we need about 50 animals for the readout per experiment. This would amount to 50 * 5 * 5 (animals/experiment * experiments/year * years) = **1250 animals**. We estimate that 1 out of 5

experiments use in utero electroporation, i.e. 250 animals. Based on previous experience, we obtain

about 3 usable electroporated pups per mother. We would thus require 250 / 3 = **83 mothers**. The requested number of animals is thus 1250 + 83 = **total 1333**.

At the start of an experiment, we will carefully determine the number of animals needed with a power-analysis, or we will start with a pilot-experiment. We will stop the experiments once the aims of the experiment have been achieved and not use further mice.

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.

(Text is identical to Appendix 3.4.4.1.)

Replacement
To disentangle the brain mechanisms that underlie plasticity in the mammalian visual system, we cannot use cell cultures or animal species with a very different visual system such as other vertebrates that lack a cerebral cortex. Intact brains of awake animals need to be studied to understand how brain activity underlies visual perception and learning. We can only study the cellular level and, in particular, the contribution of specific cells types (e.g. the interneurons) and changes in the connectivity that result from plasticity in mice due to the powerful methods that exist in this animal model (e.g. calcium imaging, 2-photon imaging, optogenetics, transgenic animals). The possibilities for invasive measurements in the human brain are limited. Furthermore, there are no models available with enough level of accurate detail to answer the same questions. Hence, the replacement of the mice needed for these experiments, is not possible.

Reduction
We reduce the number of animals to the minimum possible. First, we will calculate the number of animals needed to achieve a certain statistical power beforehand, in case we know the effect size. If the effect size is unknown, we will first do a pilot experiment using only a few animals to determine the expected effect size. Second, we will use both male and female mice whenever possible to reduce the number of surplus animals. Third, whenever possible we use animals for both in vivo and ex vivo studies.

Refinement
We use a collection of the newest genetic techniques to make our experimental manipulations as specific as possible. Furthermore, all surgeries will be carried out by persons who are well trained. Animals are housed in cages with cage enrichment in order to keep them engaged which we believe to reduce discomfort of living in a cage and improve cognitive capabilities.

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

(Text in black is identical to Appendix 3.4.4.1. except for addition in red)

Animals will be housed socially, unless it is impossible because of the risk of damage to a specific cranial

implant or eye suture. Our experience shows that mice with simple cranial windows and head posts can continue to be housed socially.

During surgeries, analgesics and anaesthesia are used to minimize pain and suffering. Breathing and temperature will be registered and level of anaesthesia and warmth of heating-pad will be adjusted as such. Peri-surgical analgesics will be administered if necessary and animals will wake up in a warm environment. Care will be taken to facilitate easy access to food and water. Mice will be allowed to recover for at least two days following surgery. Behaviour, wound area, weight and appearance will be monitored daily at least for 3 days post-surgery and longer for surgeries that would have a longer chance of complications.

Animals under fluid control are monitored very closely as described in more detail in section I.

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.

(Text is identical to Appendix 3.4.4.1.)

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.

(Text is identical to Appendix 3.4.4.1)

For some cranial implants, it may be necessary to house mice solitarily after a surgery or brain injection to prevent cage mates damaging surgical wounds or the 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. Mice cage will be enriched by bedding material and also running wheels, pipes and/or shelters when possible.

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.

(As in Appendix 3.4.4.1)

During surgery, we will use adequate anaesthesia and analgesia. Also post-surgery analgesics will be administered. Furthermore, before killing any mice, they will be deeply anesthetized.

I. Other aspects compromising the welfare of the animals

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

(Text in black is identical to Appendix 3.4.4.1., in red are additions)

Infections.
In rare cases there is a possibility of infection around the wound area. In these cases, we will apply additional analgesics and/or antibiotics. Visible signs of pathogenesis will be monitored. The following will be considered as signs of an unhealthy state of the animal: aberrant behaviour, dehydration, weight loss, nose and mouth discharge.

Insufficient recovery after surgery.
Applicable if an animal shows weight loss (more than 15% of its post-surgery weight within two days, or more than 20% in total). This occurs infrequently (<2%).

Head post detachment.
If the head post breaks off, the animal is immediately anesthetized to allow assessment of the discomfort level. If the head post becomes loose/detached due to bad adhesion to the skull we are able to reattach the head post in an immediate repair surgery. The repair surgery is performed under anaesthesia and with analgesia in an identical fashion to the original attachment surgery, with the exception that the skin does not need to be cut, this makes the repair surgery both shorter in duration and less invasive than the original implantation surgery. We estimate the discomfort to be moderate during recovery from the anaesthesia (1 day) becoming mild for 1-2 days. In the rare case (<2%) that the detachment of the head post damages the skull, the mouse is immediately euthanized under anaesthesia.

Reopening of craniotomy.
In rare cases (<5%), the craniotomy is reopened causing moderate discomfort. In these cases the mouse is immediately euthanized under anaesthesia.

Dehydration.
Animals undergoing perceptual learning do not get water ad libitum. This could result in signs of dehydration.

Explain why these effects may emerge.

(Text in black is identical to Appendix 3.4.4.1., in red are additions)

Mild to moderate discomfort due to unintended effects as described above can occur in a small percentage of mice. The head post can become loose due to the forces exerted by the mouse on the head post while fixed in the set-up. Dehydration can occur when the mouse performs poorly during training and the amount of fluid gained during training or given on non-working days is less than what is naturally lost.

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

(Text in black is identical to Appendix 3.4.4.1., in red are additions)

Each animal that undergoes surgery will be monitored for clinical parameters. The mice will be monitored for their general appearance and activity level. If a mouse has an appearance that gives cause for concern (e.g. signs of infection around the wound, hair standing up or sunken flanks, reduction in activity level) then we will notify the Animal Welfare Board and evaluate the animal together with the

veterinarian of the NIN or the animal welfare officer. Similarly, if the animal does not recover well from anesthesia we will evaluate the animal together with the veterinarian or the animal welfare officer.
In addition, the weight of the animal will be monitored and if the animal loses 15% of their weight in two consecutive days or if the animal loses more than 20% of their weight throughout the course of the experiment then the veterinarian or animal welfare officer will be contacted and a decision will be made whether a humane endpoint has been reached. If the headpost breaks off damages the skull or reopening of the craniotomy occurs, the mouse will be immediately euthanized.

While an animal is under fluid control we monitor its appearance and behaviour every day. We weigh the mouse before and after training and compare the weight to the average weight during the last week. Should the weight decrease below 90% of this weekly average we take the mouse off fluid control and give it ad libitum access to water. The weight is also checked over longer intervals to prevent a slow loss of weight. Should we notice such a slow loss we will make sure the mouse drinks more fluid by adapting task difficulty and/or substituting with gel. Additionally, we check the mouse for its general appearance and its behaviour. In our experience, the measures described do not cause any signs of dehydration such as reduced skin tension, sunken eyes or either increased or reduced activity. If any of these welfare criteria are abnormal the mouse is taken out of training and provided with ad libitum access to fluid until it has recovered. In that case, the IvD will be informed so that they can check the animal. All criteria (weight, fluid consumed per day, appearance) are logged for each individual mouse so that the history is always accessible.

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.

(Text is identical to Appendix 3.4.4.1.)

Each animal that undergoes surgery will be monitored for clinical parameters. The mice will be monitored for their general appearance and activity level. If a mouse has an appearance that gives cause for concern (e.g. signs of infection around the wound, hair standing up or sunken flanks, reduction in activity level) then we will notify the IvD. Similarly, if the animal does not recover well from anesthesia we will evaluate the animal together with the IvD.
In addition, the weight of the animal will be monitored and if the animal loses 15% of their weight in two consecutive days or if the animal loses more than 20% of their weight throughout the course of the experiment then the IvD will be contacted and a decision will be made whether a humane endpoint has been reached. If the head post breaks off damages the skull or reopening of the craniotomy occurs, the mouse will be immediately euthanized.

Indicate the likely incidence.

(Text is identical to Appendix 3.4.4.1.)

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').

The cumulative discomfort for the animals that have only undergone the unsupervised learning paradigm with no further discomfort except acute readout is **mild**. We expect that one of the five estimated experiments per year involves only unsupervised learning (250 mice = 20%)

For all other mice in this appendix (1000 mice = 80%) and for the mothers the cumulative discomfort is **moderate**.

The classification of severity of the individual procedures is described in detail in Appendix 3.4.4.1, and is

not repeated here. Three classifications are added below.

2c. Perceptual learning
Mild discomfort (entire period) due to fluid control. The discomfort is mild given that many measures are taken (described above) that ensure that the mice will receive their daily need for water.
Mild discomfort (training sessions) for head-fixation.

2d. Fear learning
Mild discomfort for sessions with noxious stimuli or with visual stimuli that have been associated with noxious stimuli.
Mild discomfort (training sessions) for head-fixation.

2e. Unsupervised learning
Mild discomfort if visual stimuli are shown that lead to a freezing or fleeing response, such as looming objects.
Mild discomfort (training sessions) for head-fixation.

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.

(As in Appendix 3.4.4.1.)

For ex vivo histology or molecular analysis, or for slice physiology it is necessary to kill the animal and extract the brain.

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'.	80100-KNAW	
1.2	Provide the name of the licenced establishment.	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 3.4.4.4	Type of animal procedure **Readout during visual learning**: Chronic in vivo assessment of neuronal function or anatomy in control mice and mice undergoing plasticity induction by visual learning

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 aim of this procedure is to measure the effect on neural structure and function by an experimental manipulation in mice during and after plasticity induced by visual learning.

The general design has the following components:
1. Instrumentalization of the experimental animal
2. Plasticity paradigm - visual learning
3. Experimental manipulation
4. Readout – chronic and acute

These components are described in detail in Appendix 3.4.4.3, and are not repeated here.
Added here is chronic readout:

4. Readout – Chronic

We want to observe the changes in the brain while plasticity takes places. For this reason we would like to record and image the structure and functioning of visual system during plasticity. To obtain a baseline of the dynamics will we commence measuring before plasticity induction and continue measuring after the period of visual plasticity. Chronic readout consist of one or both of the following procedures:
4f. Chronic in vivo probe recording, repeated measurements in awake or anaesthetised mice using a recording probe, e.g. an extracellular electrode, an intracellular pipet, or an electrochemical probe taking place over multiple sessions.

4g. **Chronic in vivo imaging**, repeated optical measurements in awake or anaesthetised mice of brain structure and activity over multiple sessions. Examples are imaging of GFP in a selection of neurons, genetically coded calcium indicators, or wide-field intrinsic signal imaging.

The primary outcome parameters of these chronic recording and imaging experiments are the dynamics in the changes in neuronal spiking or synaptic activity of specific groups of neurons induced by the visual plasticity induction paradigm while manipulation the brain.

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

The procedures are described in detail in Appendix 3.4.4.1, and are not repeated here.
Two procedures are added:

For all experiments, the cranial surgery will have taken place multiple days or weeks before the first recording and imaging session. Typically, we will record or image for less than 10 sessions. In some cases, however, it may be that plasticity continues to change the visual system. In these cases, we may record more sessions, but we do not expect that ever more than 20 sessions will be useful.
The measurements can be combined, and therefore the percentages for frequency of the procedures add up to above 100%.

Procedure 4f. Chronic in vivo probe recording (procedure for 75% of the mice)
In multiple sessions, we will record brain activity using an invasive probe, such as an extracellular microelectrode, intracellular pipet or electrochemical probe. The recording session can start with induction of anaesthesia, but in the majority of cases will be in an awake animal. Awake recording can be done in head-fixed animals or freely moving animals using a tethered or wireless connection. For head-fixed awake sessions, mice will have been habituated to being head-fixed as part of the cranial surgery procedure 1d.

Procedure 4g. Chronic in vivo imaging. (procedure for 75% of the mice)
In multiple sessions (possibly combined with procedure 4f), we will a microscope or a macroscope (low magnification imaging setup) to image structure and activity of the brain using, for instance, intrinsic signal, flavoprotein, fluorescent dyes or fluorescent proteins. The imaging session can start with induction of anaesthesia, especially for structural imaging, but can also be in an awake animal, especially for functional imaging. Awake imaging can be done in head-fixed animals or freely moving animals using optical fibres or miniature microscopes. For head-fixed awake sessions, mice will have been habituated to being head-fixed as part of the cranial surgery procedure 1d.

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

(Text in black is identical to Appendix 3.4.4.1. In red are the differences.)

Pilot experiments
Statistical methods do not apply. Establishing new manipulations requires testing and adaptation based on obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Initial 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. By taking multiple longitudinal measurements in the same mice, we can do paired statistics which will have more power than comparing mice undergoing visual plasticity with other mice not undergoing plasticity.

B. The animals

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

(Text in black is identical to Appendix 3.4.4.2. In red are the differences.)

Species and origin

We will use mice (Mus musculus) in these experiments. This choice is based on the amount of fundamental knowledge that exists about the anatomy and physiology of the mouse brain and the availability of sophisticated technologies for investigating brain mechanisms, including a variety of genetically engineered strains. We include different mouse lines, including wild-type (e.g. C57Bl6) and genetically modified animals. The choice of transgenic animals depends on the specific research question. For example, if we need to measure activity of specific neurons, we can make use of transgenic mouse line that expresses, for example, a calcium indicator in a specific subclass of interneurons. We will carefully consider the best mouse model for every research question separately, taking latest developments into account. All mice are derived from the NIN, an establishment licensed by the NVWA, or from a registered commercial company.

In the animals in which variants of endogenous proteins are expressed or knocked-out it may be possible that there is discomfort as a result of an affected phenotype, but no discomfort is reported for any of the lines that we intend to use. For this protocol we assume that there is no congenic discomfort for the breeding of any of the lines. New lines will be monitored for two generations and the outcome will be presented to the IvD. In the unlikely event we want to use a strain with congenital discomfort we will add an addendum for such a mouse line to the CCD protocol.

Sex

For readout, we will use both males and females from the animal lines that are bred in-house, to reduce the number of surplus animals. For most of our questions in our line of research (plasticity in the visual system) we do not expect any differences between genders. However, we will document the gender and investigate if there are any gender differences that are relevant for our research questions. If so, we will ask for approval of the IVD to only use mice of the appropriate sex for such experiments.

Life stages

We will only do experiments involving visual stimulation in animals after eye opening. Mice open their eyes not before postnatal day P10, but manipulations may need to be done earlier, e.g. transfection takes several days after viral injection. The majority of mice will consist of adults and juveniles after weening at P21. In mice, vision deteriorates with age, so most experiments will be in mice that are less than 1 year old. In utero electroporation will take place in embryos not younger than E10. Mothers used for in utero electroporation will be adult.

Animal number

The estimate of the total number of experimental groups is primarily based on our experience over the past years performing very similar experiments. There are about a dozen researchers in our laboratories. We expect them together to do about 5 separate experiments (e.g. the role of a specific gene in plasticity in a visual area) with this procedure per year. Based on previous experiments over the last decade, we estimate that we need about 30 animals for the readout per experiment. This is less than for a typical experiment where we only take an acute measurement, because the longitudinal measurements have more power in a significance calculation. This would amount to 30 * 5 * 5 (animals/experiment * experiments/year * years) = **750 animals**. We estimate that 1 out of 5 experiments use in utero electroporation, i.e. 150 animals. Based on previous experience, we obtain about 3 usable electroporated pups per mother. We would thus require 150 / 3 = **50 mothers**. The requested number of animals is thus 750 + 50 = **total 800**.

At the start of an experiment, we will carefully determine the number of animals needed with a power-analysis, or we will start with a pilot-experiment. We will stop the experiments once the aims of the experiment have been achieved and not use further mice.

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.

(Text in black is identical to Appendix 3.4.4.1. In red are the differences.)

Replacement
To disentangle the brain mechanisms that underlie plasticity in the mammalian visual system, we cannot use cell cultures or animal species with a very different visual system such as other vertebrates that lack a cerebral cortex. Intact brains of awake animals need to be studied to understand how brain activity underlies visual perception and learning. We can only study the cellular level and, in particular, the contribution of specific cells types (e.g. the interneurons) and changes in the connectivity that result from plasticity in mice due to the powerful methods that exist in this animal model (e.g. calcium imaging, 2-photon imaging, optogenetics, transgenic animals). The possibilities for invasive measurements in the human brain are limited. Furthermore, there are no models available with enough level of accurate detail to answer the same questions. Hence, the replacement of the mice needed for these experiments, is not possible.

Reduction
We reduce the number of animals to the minimum possible. First, we will calculate the number of animals needed to achieve a certain statistical power beforehand, in case we know the effect size. If the effect size is unknown, we will first do a pilot experiment using only a few animals to determine the expected effect size. Second, we will use both male and female mice whenever possible to reduce the number of surplus animals. Third, whenever possible we use animals for both in vivo and ex vivo studies. Fourth, by taking repeated longitudinal measurements in one animal, we reduce the number of animals needed.

Refinement
We use a collection of the newest genetic techniques to make our experimental manipulations as specific as possible. Furthermore, all surgeries will be carried out by persons who are well trained. Animals are housed in cages with cage enrichment in order to keep them engaged which we believe to reduce discomfort of living in a cage and improve cognitive capabilities.

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

(Text is identical to Appendix 3.4.4.3.)

Animals will be housed socially, unless it is impossible because of the risk of damage to a specific cranial implant or eye suture. Our experience shows that mice with simple cranial windows and head posts can continue to be housed socially.

During surgeries, analgesics and anaesthesia are used to minimize pain and suffering. Breathing and temperature will be registered and level of anaesthesia and warmth of heating-pad will be adjusted as such. Peri-surgical analgesics will be administered if necessary and animals will wake up in a warm environment. Care will be taken to facilitate easy access to food and water. Mice will be allowed to

recover for at least two days following surgery. Behaviour, wound area, weight and appearance will be monitored daily at least for 3 days post-surgery and longer for surgeries that would have a longer chance of complications.

Animals under fluid control are monitored very closely as described in more detail in section I.

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.

(Text is identical to Appendix 3.4.4.1.)

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.

(Text is identical to Appendix 3.4.4.1.)

For some cranial implants, it may be necessary to house mice solitarily after a surgery or brain injection to prevent cage mates damaging surgical wounds or the 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. Mice cage will be enriched by bedding material and also running wheels, pipes and/or shelters when possible.

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.

(Text is identical to Appendix 3.4.4.1.)
During surgery, we will use adequate anaesthesia and analgesia. Also post-surgery analgesics will be

administered. Furthermore, before killing any mice, they will be deeply anesthetized.

I. Other aspects compromising the welfare of the animals

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

(Text is identical to Appendix 3.4.4.3.)

Infections.
In rare cases there is a possibility of infection around the wound area. In these cases, we will apply additional analgesics and/or antibiotics. Visible signs of pathogenesis will be monitored. The following will be considered as signs of an unhealthy state of the animal: aberrant behaviour, dehydration, weight loss, nose and mouth discharge.

Insufficient recovery after surgery.
Applicable if an animal shows weight loss (more than 15% of its post-surgery weight within two days, or more than 20% in total). This occurs infrequently (<2%).

Head post detachment.
If the head post breaks off, the animal is immediately anesthetized to allow assessment of the discomfort level. If the head post becomes loose/detached due to bad adhesion to the skull we are able to reattach the head post in an immediate repair surgery. The repair surgery is performed under anaesthesia and with analgesia in an identical fashion to the original attachment surgery, with the exception that the skin does not need to be cut, this makes the repair surgery both shorter in duration and less invasive than the original implantation surgery. We estimate the discomfort to be moderate during recovery from the anaesthesia (1 day) becoming mild for 1-2 days. In the rare case (<2%) that the detachment of the head post damages the skull, the mouse is immediately euthanized under anaesthesia.

Reopening of craniotomy.
In rare cases (<5%), the craniotomy is reopened causing moderate discomfort. In these cases the mouse is immediately euthanized under anaesthesia.

Dehydration.
Animals undergoing perceptual learning do not get water ad libitum. This could result in signs of dehydration.

Explain why these effects may emerge.

(Text is identical to Appendix 3.4.4.3.)

Mild to moderate discomfort due to unintended effects as described above can occur in a small percentage of mice. The head post can become loose due to the forces exerted by the mouse on the head post while fixed in the set-up. Dehydration can occur when the mouse performs poorly during training and the amount of fluid gained during training or given on non-working days is less than what is naturally lost.

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

(Text is identical to Appendix 3.4.4.3.)

Each animal that undergoes surgery will be monitored for clinical parameters. The mice will be monitored for their general appearance and activity level. If a mouse has an appearance that gives cause for concern (e.g. signs of infection around the wound, hair standing up or sunken flanks, reduction in activity level) then we will notify the Animal Welfare Board and evaluate the animal together with the veterinarian of the NIN or the animal welfare officer. Similarly, if the animal does not recover well from anesthesia we will evaluate the animal together with the veterinarian or the animal welfare officer.
In addition, the weight of the animal will be monitored and if the animal loses 15% of their weight in two consecutive days or if the animal loses more than 20% of their weight throughout the course of the experiment then the veterinarian or animal welfare officer will be contacted and a decision will be made whether a humane endpoint has been reached. If the head post breaks off damages the skull or reopening of the craniotomy occurs, the mouse will be immediately euthanized.

While an animal is under fluid control we monitor its appearance and behaviour every day. We weigh the mouse before and after training and compare the weight to the average weight during the last week. Should the weight decrease below 90% of this weekly average we take the mouse off fluid control and give it ad libitum access to water. The weight is also checked over longer intervals to prevent a slow loss of weight. Should we notice such a slow loss we will make sure the mouse drinks more fluid by adapting task difficulty and/or substituting with gel. Additionally, we check the mouse for its general appearance and its behaviour. In our experience, the measures described do not cause any signs of dehydration such as reduced skin tension, sunken eyes or either increased or reduced activity. If any of these welfare criteria are abnormal the mouse is taken out of training and provided with ad libitum access to fluid until it has recovered. In that case, the Animal Welfare Board will be informed so that they can check the animal. All criteria (weight, fluid consumed per day, appearance) are logged for each individual mouse so that the history is always accessible.

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.

(Text is identical to Appendix 3.4.4.1.)

Each animal that undergoes surgery will be monitored for clinical parameters. The mice will be monitored for their general appearance and activity level. If a mouse has an appearance that gives cause for concern (e.g. signs of infection around the wound, hair standing up or sunken flanks, reduction in activity level) then we will notify the IvD. Similarly, if the animal does not recover well from anesthesia we will evaluate the animal together with the IvD.
In addition, the weight of the animal will be monitored and if the animal loses 15% of their weight in two consecutive days or if the animal loses more than 20% of their weight throughout the course of the experiment then the IvD will be contacted and a decision will be made whether a humane endpoint has been reached. If the headpost breaks off damages the skull or reopening of the craniotomy occurs, the mouse will be immediately euthanized.

Indicate the likely incidence.

(Text is identical to Appendix 3.4.4.1.)

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').

Cumulative discomfort for all animals, including the mothers, is classified as **moderate**

The classification of severity of the individual procedures are described in detail in Appendix 3.4.4.3, and are not repeated here. Two classifications are added below:

4f. Chronic in vivo probe recording
Moderate discomfort due to repeated recovery from anaesthesia for recording under anaesthesia.
Moderate discomfort due to head fixation in awake recording.

4g. Chronic in vivo imaging
Moderate discomfort due to repeated recovery from anaesthesia for recording under anaesthesia.
Moderate discomfort due to head fixation in awake imaging.

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.

As in Appendix 3.4.4.3.

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'.	80100-KNAW	
1.2	Provide the name of the licenced establishment.	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 **Readout in naïve mouse:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in mice without plasticity induction

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 aim of this procedure is to measure neural structure and function and the effect of an experimental manipulation thereon in naïve mice. The goal of this can be to investigate the function of particular brain regions that processes visual information (subaim A2a of the Research strategy). The knowledge obtained with these experiments can guide our experiments for the plasticity paradigms of appendices 3.4.4.1-3.4.4.4. The measurements from this procedure can also be used as control for experiments with plasticity paradigms and acute readout (described in appendices 3.4.4.1 and 3.4.4.3).

The general design has the following components:

1. Instrumentalization of the experimental animal
2. Experimental manipulation
3. Readout – acute

These components are described in detail in Appendix 3.4.4.1. excluding the visual deprivation paradigms. Overlapping texts are not repeated here.

In addition, this group of procedures can be used for the introduction, training, and optimization of the (novel) techniques used for the instrumentalization, manipulation and read out.

The primary outcome parameters of the acute readout are the structure and function of the brain at areal, cellular and subcellular level.

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

The procedures are described in detail in Appendix 3.4.4.1, and are not repeated here.

Visual deprivation procedures are not included here.

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

(Text is identical to Appendix 3.4.4.1)

Pilot experiments
Statistical methods do not apply. Establishing new manipulations requires testing and adaptation based on obtained results. Adapted procedures are then tested in new groups, until the full procedure is established and formal experiments can start.

Initial 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.

Text in black is identical to Appendix 3.4.4.2. In red are the differences.)

Species and origin
We will use mice (Mus musculus) in these experiments. This choice is based on the amount of fundamental knowledge that exists about the anatomy and physiology of the mouse brain and the availability of sophisticated technologies for investigating brain mechanisms, including a variety of genetically engineered strains. We include different mouse lines, including wild-type (e.g. C57Bl6) and genetically modified animals. The choice of transgenic animals depends on the specific research question. For example, if we need to measure activity of specific neurons, we can make use of transgenic mouse line that expresses, for example, a calcium indicator in a specific subclass of interneurons. We will carefully consider the best mouse model for every research question separately, taking latest developments into account. All mice are derived from the NIN, an establishment licensed by the NVWA, or from a registered commercial company.
In the animals in which variants of endogenous proteins are expressed or knocked-out it may be possible that there is discomfort as a result of an affected phenotype, but no discomfort is reported for any of the lines that we intend to use. For this protocol we assume that there is no congenic discomfort for the breeding of any of the lines. New lines will be monitored for two generations and the outcome will be presented to the IvD. In the unlikely event we want to use a strain with congenital discomfort we will add an addendum for such a mouse line to the CCD protocol.

Sex
For readout, we will use both males and females from the animal lines that are bred in-house, to reduce the number of surplus animals. For most of our questions in our line of research (plasticity in the visual system) we do not expect any differences between genders. However, we will document the gender and investigate if there are any gender differences that are relevant for our research questions. If so, we will ask for approval of the IVD to only use mice of the appropriate sex for such experiments.

Life stages
We will only do experiments involving visual stimulation in animals after eye opening. Mice open their eyes not before postnatal day P10, but manipulations may need to be done earlier, e.g. transfection takes several days after viral injection. The majority of mice will consist of adults and juveniles after weening at P21. In mice, vision deteriorates with age, so most experiments will be in mice that are less than 1 year old. In utero electroporation will take place in embryos not younger than E10. Mothers used for in utero electroporation will be adult.

Animal number
The animals in this group can act as controls for the plasticity paradigms with acute readout (appendices 3.4.4.1 and 3.4.4.3). The total number for these groups is **2500 readout animals** and **167 mothers** for in utero electroporation. The motivation of this number (**2667 mice**) is given in 3.4.4.1. and 3.4.4.3.

In addition, we also expect to do about 4 separate experiments each year, with only acute readout after anaesthesia induction or with only ex vivo analysis, and where we do not need in utero electroporation or procedures with moderate discomfort. Based on previous experiments over the last decade, we estimate that we need about 30 animals for the readout per experiment. This would amount to 30 * 4 * 5 (animals/experiment * experiments/year * years) = **600 animals**.

> For appendix 3.4.4.5 **total 3267** animals

At the start of an experiment, we will carefully determine the number of animals needed with a power-analysis, or we will start with a pilot-experiment. We will stop the experiments once the aims of the experiment have been achieved and not use further mice.

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.

(Text is identical to Appendix 3.4.4.1.)

Replacement
To disentangle the brain mechanisms that underlie plasticity in the mammalian visual system, we cannot use cell cultures or animal species with a very different visual system such as other vertebrates that lack a cerebral cortex. Intact brains of awake animals need to be studied to understand how brain activity underlies visual perception and learning. We can only study the cellular level and, in particular, the contribution of specific cells types (e.g. the interneurons) and changes in the connectivity that result from plasticity in mice due to the powerful methods that exist in this animal model (e.g. calcium imaging, 2-photon imaging, optogenetics, transgenic animals). The possibilities for invasive measurements in the human brain are limited. Furthermore, there are no models available with enough level of accurate detail to answer the same questions. Hence, the replacement of the mice needed for these experiments, is not possible.

Reduction
We reduce the number of animals to the minimum possible. First, we will calculate the number of animals needed to achieve a certain statistical power beforehand, in case we know the effect size. If the effect size is unknown, we will first do a pilot experiment using only a few animals to determine the expected effect size. Second, we will use both male and female mice whenever possible to reduce the number of surplus animals. Third, whenever possible we use animals for both in vivo and ex vivo studies.

Refinement
We use a collection of the newest genetic techniques to make our experimental manipulations as specific as possible. Furthermore, all surgeries will be carried out by persons who are well trained. Animals are housed in cages with cage enrichment in order to keep them engaged which we believe to reduce discomfort of living in a cage and improve cognitive capabilities.

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

(Text is identical to Appendix 3.4.4.1.)

Animals will be housed socially, unless it is impossible because of the risk of damage to a specific cranial implant or eye suture. Our experience shows that mice with simple cranial windows and head posts can continue to be housed socially.

During surgeries, analgesics and anaesthesia are used to minimize pain and suffering. Breathing and temperature will be registered and level of anaesthesia and warmth of heating-pad will be adjusted as such. Peri-surgical analgesics will be administered if necessary and animals will wake up in a warm environment. Care will be taken to facilitate easy access to food and water. Mice will be allowed to recover for at least two days following surgery. Behaviour, wound area, weight and appearance will be monitored daily at least for 3 days post-surgery and longer for surgeries that would have a longer chance of complications.

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.

(Text is identical to Appendix 3.4.4.1.)

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.

(Text is identical to Appendix 3.4.4.1.)

For some cranial implants, it may be necessary to house mice solitarily after a surgery or brain injection to prevent cage mates damaging surgical wounds or the 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. Mice cage will be enriched by bedding material and also running wheels, pipes and/or shelters when possible.

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.

(Text is identical to Appendix 3.4.4.1.)

During surgery, we will use adequate anaesthesia and analgesia. Also post-surgery analgesics will be administered. Furthermore, before killing any mice, they will be deeply anesthetized.

I. Other aspects compromising the welfare of the animals

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

(Text is identical to Appendix 3.4.4.1.)

Infections.
In rare cases there is a possibility of infection around the wound area. In these cases, we will apply additional analgesics and/or antibiotics. Visible signs of pathogenesis will be monitored. The following will be considered as signs of an unhealthy state of the animal: aberrant behaviour, dehydration, weight loss, nose and mouth discharge.

Insufficient recovery after surgery.
Applicable if an animal shows weight loss (more than 15% of its post-surgery weight within two days, or more than 20% in total). This occurs infrequently (<2%).

Head post detachment.
If the head post breaks off, the animal is immediately anesthetized to allow assessment of the discomfort level. If the head post becomes loose/detached due to bad adhesion to the skull we are able to reattach the head post in an immediate repair surgery. The repair surgery is performed under anaesthesia and with analgesia in an identical fashion to the original attachment surgery, with the exception that the skin does not need to be cut, this makes the repair surgery both shorter in duration and less invasive than the original implantation surgery. We estimate the discomfort to be moderate during recovery from the anaesthesia (1 day) becoming mild for 1-2 days. In the rare case (<2%) that the detachment of the head post damages the skull, the mouse is immediately euthanized under anaesthesia.

Reopening of craniotomy.
In rare cases (<5%), the craniotomy is reopened causing moderate discomfort. In these cases the mouse is immediately euthanized under anaesthesia.

Explain why these effects may emerge.

(Text is identical to Appendix 3.4.4.1.)

Mild to moderate discomfort due to unintended effects as described above can occur in a small percentage of mice. The head post can become loose due to the forces exerted by the mouse on the head post while fixed in the set-up.

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

(Text is identical to Appendix 3.4.4.1.)

We monitor the animal's behaviour, wound area and physiology in these days after surgery. The surgeries are performed using adequate surgical procedures in semi-sterile conditions and without unnecessary delays to minimize the amount of time the animal is under anaesthesia. Animals will be monitored daily and if adverse effects are present, this will be discussed with the IVD and/or veterinary officer. If necessary, treatment will be initiated (topically or systemically applied medication).

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.

(Text is identical to Appendix 3.4.4.1.)

Each animal that undergoes surgery will be monitored for clinical parameters. The mice will be monitored for their general appearance and activity level. If a mouse has an appearance that gives cause for concern (e.g. signs of infection around the wound, hair standing up or sunken flanks, reduction in activity level) then we will notify the IvD. Similarly, if the animal does not recover well from anesthesia we will evaluate the animal together with the IvD.
In addition, the weight of the animal will be monitored and if the animal loses 15% of their weight in two consecutive days or if the animal loses more than 20% of their weight throughout the course of the experiment then the IvD will be contacted and a decision will be made whether a humane endpoint has been reached. If the headpost breaks off damages the skull or reopening of the craniotomy occurs, the mouse will be immediately euthanized.

Indicate the likely incidence.

(Text is identical to Appendix 3.4.4.1.)

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').

The cumulative discomfort for the animals that have only acute readout is **mild** (600 mice).
For all other mice in this appendix (2667, including mothers) the cumulative discomfort is **moderate**.

The classification of severity of the individual procedures is described in detail in Appendix 3.4.4.1, and is not repeated here.
Plasticity paradigm procedures are not included in this Appendix.

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.

(Text is identical to Appendix 3.4.4.1.)

For ex vivo histology or molecular analysis, or for slice physiology it is necessary to kill the animal and extract the brain.

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

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de Praktische Handreiking: Ethisch Toetsingskader voor proefdiergebruik. Voor voorbeelden, zie bijlage I.

Herhaling van antwoorden is niet nodig. Indien van toepassing kan verwezen worden naar een bij een eerdere vraag verstrekt antwoord.

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD80100 2017 1045

2. Titel van het project: Plasticity in the visual system and its regulation

3. Titel van de NTS: De werking en regulatie van leren in het visuele systeem

4. Type aanvraag:
   - ■ nieuwe aanvraag projectvergunning
   - □ wijziging van vergunning met nummer

5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: [redacted]
   - e-mailadres contactpersoon: [redacted]

6. Adviestraject (data dd-mm-jjjj):
   - ■ ontvangen door DEC: 15-02-2017
   - ■ aanvraag compleet: 15-03-2017
   - ■ in vergadering besproken: 23-02-2017 en 23-03-2017
   - ■ anderszins behandeld: niet van toepassing
   - ■ termijnonderbreking(en) van 27-02-2017 tot 15-03-2017
   - ■ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: niet van toepassing
   - ■ aanpassing aanvraag: finale herziene versie ontvangen op 15-03-2017
   - ■ advies aan CCD: 04-04-2017

7. Geef aan of de aanvraag is afgestemd met de IvD en deze de instemming heeft van de IvD.
   De IvD geeft aan dat de aanvrager de aanvraag met de IvD heeft afgestemd en dat de aanvraag de instemming heeft van de IvD[redacted]

Bij de punten 8 t/m 10 kan worden volstaan met 'n.v.t.' wanneer de betreffende acties niet aan de orde zijn geweest. Bij vragen die gericht zijn op het compleet maken van de aanvraag (aanvullingen achtergrond informatie etc) kan bij punten 8 en 9 worden volstaan met de vermelding van het type vragen en de vermelding dat de aanvraag op de desbetreffende onderdelen is aangepast of dat de antwoorden in de aanvraag zijn verwerkt. Bij vragen die gericht zijn op het verkrijgen van verklaringen voor keuzes die door de aanvrager gemaakt worden, kan niet worden volstaan met het weergeven van de strekking van de antwoorden tenzij de antwoorden volledig in de aanvraag zijn opgenomen. Als dat het geval is, moet dat in het DEC advies worden benoemd en in de aanvraag inzichtelijk worden gemaakt.

8. Eventueel horen van aanvrager:
   - Datum: 23-02-2017
   - Plaats: Amsterdam
   - Aantal aanwezige DEC-leden: 6
   - Aanwezige (namens) aanvrager: de hoofdaanvrager
   - Gestelde vraag / vragen: de mondeling gestelde vragen zijn later tevens schriftelijk aan de aanvrager gestuurd en hebben geleid tot aanpassing van de aanvraag
   - Verstrekt(e) antwoord(en): zie onder vraag 9
     Het horen van de aanvrager heeft wel/niet geleid tot aanpassing van de aanvraag: wel; zie onder vraag 9

9. Correspondentie met de aanvrager
   - Datum: 27-02-2017; n.a.v. de bespreking van de eerste versie van de aanvraag
   - Gestelde vraag/vragen: Aanvullende vragen ter completering van de aanvraag. De vragen waren voornamelijk gericht om meer duidelijkheid te verkrijgen over de relatie van de verschillende plasticiteitsmodellen en de doelstelling van het project. Gezien de aard van de vragen was de DEC van mening dat de herziene versie van het project opnieuw moest worden besproken tijdens de volgende DEC vergadering op 23-03-2017.
   - Datum antwoord: 15-03-2017
   - Verstrekt(e) antwoord(en): De aanvrager heeft de aanvraag gecomplementeerd en de gevraagde aanpassingen zijn doorgevoerd in de finale versie. Deze versie en de antwoorden zijn besproken door de DEC op 23-03-2017 en de initiële zorgen van de DEC zijn weggenomen in de finale versie.

   - De antwoorden hebben wel/niet geleid tot aanpassing van de aanvraag: wel

10. Eventuele adviezen door experts (niet lid van de DEC): niet van toepassing
    - Aard expertise
    - Deskundigheid expert
    - Datum verzoek
    - Strekking van het verzoek
    - Datum expert advies
    - Advies expert

B. Beoordeling (adviesvraag en behandeling)

1. Is het project vergunningplichtig (dierproeven in de zin der wet)? Indien van toepassing, licht toe waarom het project niet vergunningplichtig is en of daar discussie over geweest is.
   Indien niet vergunningplichtig, ga verder met onderdeel E. Advies.
   Het project is vergunningplichtig.

2. De aanvraag betreft een nieuwe aanvraag / een wijziging op een bestaande vergunning.
   Nieuwe aanvraag – Zie A4

3. Is de DEC competent om hierover te adviseren?
   Ja

4. Geef aan of DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, zijn uitgesloten van de behandeling van de aanvraag en het opstellen van het advies. Indien van toepassing, licht toe waarom.
   Er zijn geen DEC-leden uitgesloten van de behandeling en het opstellen van het advies.

C. Beoordeling (inhoud)

1. Beoordeel of de aanvraag toetsbaar is en voldoende samenhang heeft *(Zie handreiking 'Invulling definitie project'; zie bijlage I voor toelichting en voorbeeld).*
   Deze aanvraag heeft een concrete en duidelijke hoofddoelstelling, te weten: het verkrijgen van inzichten hoe verschillende types plasticiteit in het visueel systeem tot stand komen via veranderingen in feedforward en feedback verbindingen en hoe deze veranderingen zijn gereguleerd. Het hoofddoel zal worden bereikt door het verrichten van studies naar de manier waarop ervarings-afhankelijke veranderingen van hersenfuncties, plasticiteit, worden bewerkstelligd door herschikkingen van neuronale verbindingen (subdoel 1) en wat de cellulaire en moleculaire mechanismen zijn die de mate van plasticiteit reguleren (subdoel 2).

   De twee subdoelen van het project bestaan uit verschillende opeenvolgende fases die elk in voldoende mate zijn uitgewerkt in de beschrijving van de strategie onder 3.4.1 van het voorstel. De DEC komt tot de conclusie dat de aanvraag overeen komt met voorbeeld 1 van de handreiking 'Invulling definitie project'. De proeven voor de individuele subdoelen vertonen een logische tijds- en uitkomstafhankelijke opeenvolging en zijn verbonden door duidelijke go/no go momenten. Het bereiken van de subdoelen leidt uiteindelijk tot het bereiken van het hoofddoel. De aanvraag is naar de mening van de DEC te typeren als een project. De aanvraag omvat een hoofddoel en twee subdoelen die elk in voldoende mate zijn uitgewerkt. De subdoelen hebben geen onderlinge relatie of tijdsafhankelijkheid en zullen onafhankelijk en parallel aan elkaar uitgevoerd worden. De DEC is van mening dat de aanvraag overeen komt met voorbeeld 4B van de handreiking 'Invulling definitie project' en dus voldoet aan de definitie Project.

   Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan. De DEC is er daardoor van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en dat er niet onnodig dieren gebruikt zullen worden.

   Gezien het bovenstaande komt de DEC tot de conclusie dat de aanvraag voldoende samenhang heeft en daarmee toetsbaar is.

2. Signaleer of er mogelijk tegenstrijdige wetgeving is die het uitvoeren van de proef in de weg zou kunnen staan. Het gaat hier om wetgeving die gericht is op de gezondheid en welzijn van het dier of het voortbestaan van de soort (bijvoorbeeld Wet dieren en Wet Natuurbescherming).
   Dit valt buiten de taakstelling van de DEC als beschreven in artikel 18a.2.b van de Wod. Naar deze specifieke informatie wordt in het aanvraagformulier en de bijbehorende toelichting niet gevraagd en de aanvrager heeft deze informatie dan ook niet verstrekt. Het is voor de DEC daarom niet mogelijk om op dit punt een onderbouwde uitspraak te doen. De DEC wil erop wijzen dat mocht dit in sommige omstandigheden wel het geval zijn dat de CCD in een procedure voorziet waarin de aanvrager inzage krijgt en verweer kan voeren.

3. Beoordeel of de in de projectaanvraag aangekruiste doelcategorie(ën) aansluit(en) bij de hoofddoelstelling. Nevendoelstellingen van beperkt belang hoeven niet te worden aangekruist in het projectvoorstel.
   De doelcategorie sluit aan bij de hoofddoelstelling.

Belangen en waarden

4. Benoem zowel het directe doel als het uiteindelijke doel en geef aan of er een directe en reële relatie is tussen beide doelstellingen. Beoordeel of het directe doel gerechtvaardigd is binnen de context van het onderzoeksveld *(Zie Praktische handreiking ETK: Stap 1.C4; zie bijlage I voor voorbeeld)*.
   Het *directe doel* van het project is het verkrijgen van inzichten hoe verschillende types plasticiteit in het visueel systeem tot stand komen via veranderingen in feedforward en feedback verbindingen en hoe deze veranderingen zijn gereguleerd. De nieuwe inzichten zullen kunnen bijdragen aan het verbeteren van therapieën van ziektebeelden die het resultaat zijn van abnormaliteiten in neuronale plasticiteit gedurende kritische fases van ontwikkeling van het brein (amblyopia – autisme – verstandelijke beperking). Verder kunnen de nieuwe inzichten van belang zijn om in het volwassen stadium plasticiteit tijdelijk te verhogen in het kader van regeneratieve herstel therapieën in de context van neurodegeneratie of hersentrauma.

   De DEC is ervan overtuigd dat het doel gerechtvaardigd is binnen de context van het onderzoeksveld. De aanvrager heeft duidelijk gemaakt wat de status van het onderzoeksveld is, wat de bijdrage van het al verrichte werk van de onderzoeksgroep is geweest, en wat de bijdrage van dit project aan het onderzoeksveld naar verwachting zal zijn. Uit de aanvraag blijkt dat de fundamenteel wetenschappelijke kennis over plasticiteit, en de regulatie daarvan, nog beperkt is.

   Het directe wetenschappelijke belang van de resultaten is naar opvatting van de DEC groot.

5. Benoem de belanghebbenden in het project en beschrijf voor elk van de belanghebbenden welke morele waarden in het geding zijn of bevorderd worden *(Zie Praktische handreiking ETK: Stap 2.B en tabel 1; zie bijlage I voor voorbeeld)*
   De belangrijkste belanghebbenden in dit onderzoeksproject zijn: (i) *De proefdieren.* De integriteit van de dieren zal in geringe mate worden aangetast. Het merendeel van de dieren (89%) zal matig ongerief ondervinden door de proeven en een kleiner deel (11%) licht ongerief. In het kader van de proeven zullen de dieren worden gedood. De dieren hebben er belang bij om gevrijwaard te blijven van ongerief en doding. (ii) *De bij de uitvoering van het project betrokken onderzoekers.* De onderzoekers zullen een substantiële toename in kennis en vaardigheden verkrijgen. De carrièremogelijkheden van de onderzoekers zullen verbeteren door publicaties. Ook de kans op het behouden en verkrijgen van nieuwe onderzoeksmogelijkheden, veelal deels gebaseerd op een goede wetenschappelijke reputatie, zal toenemen. Deze waarden zijn naar opvatting van de DEC echter van gering gewicht in de ethische afweging. (iii) *Onderzoekers in veld van de neurobiologie.* Dit onderzoek is in de eerste plaats fundamenteel van aard. Het zal resulteren in een toename van de neurobiologische inzichten hoe verschillende types plasticiteit in het visueel systeem tot stand komen via veranderingen in feedforward en feedback verbindingen en hoe deze veranderingen zijn gereguleerd. (iv) *De doelgroepen in de maatschappij.* Deze kennis draagt bij aan inzichten in het ontstaan van ziektebeelden ten gevolge van afwijkingen in neuronale plasticiteit gedurende kritische fases van ontwikkeling van het brein (amblyopia – autisme – verstandelijke beperking) en tevens wordt inzicht gekregen over de manieren waarop in het volwassen brein het niveau van plasticiteit is beperkt hetgeen herstel bij neurodegeneratieve ziektes en na hersentrauma verhinderd. Op basis van de nieuw verworven kennis kunnen nieuwe of verbeterde therapieën worden opgesteld met vooruitzichten op een succesvol herstel

   Is er aanleiding voor de DEC om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken?

   Nee

Proefopzet en haalbaarheid

6. Beoordeel of de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven voldoende gewaarborgd zijn. Licht uw beoordeling toe. *(Zie Praktische handreiking ETK: Stap 1.C5).*
   De DEC is ervan overtuigd dat de aanvrager over voldoende expertise en de geschikte voorzieningen beschikt om de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn te realiseren. De DEC is er van overtuigd dat de aanvrager voldoende expertise heeft om gedurende het project te kunnen blijven voldoen aan de 3V's.

7. Beoordeel of het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en of de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project. Licht uw beoordeling toe. *Zie Praktische handreiking ETK: Stap 1.C6)*.
   De DEC is van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen van het project en bij recente wetenschappelijke inzichten. De DEC acht het reëel om te veronderstellen dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, nieuwe en/of aanvullende fundamenteel wetenschappelijke kennis zal worden verkregen en dat de gekozen strategie en experimentele aanpak zal leiden tot het behalen van de doelstelling van het project. De gevraagde looptijd van 5 jaar acht de DEC reëel gezien de opbouw, de grootte van de onderzoeksgroep en de financiële ondersteuning van NWO en de Europese Unie.
   Tijdens de uitvoering van het project zullen de in de aanvraag beschreven kaders, inclusief de kaders van ongerief, nauwgezet door de IvD bewaakt worden.
   De keuze voor het gebruik van de muis als proefdier en het gebruik van de vijf verschillende Type Dierproeven is duidelijk en in goed onderbouwd.
   Daarnaast zijn de gegevens uit het vooronderzoek verkregen in de muis en biedt de continuering van het onderzoek aan dezelfde proefdiersoort een grotere kans op het behalen van de doelstellingen.

Welzijn dieren

8. Geef aan of er sprake is van één of meerdere bijzondere categorieën van dieren, omstandigheden of behandeling van de dieren. Beoordeel of de keuze hiervoor voldoende wetenschappelijk is onderbouwd en of de aanvrager voldoet aan de in de Wet op de Dierproeven (Wod). voor de desbetreffende categorie genoemde beperkende voorwaarden. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C1; zie bijlage I voor toelichting en voorbeelden).*

   ☐ Bedreigde diersoort(en) (10e, lid 4)
   ☐ Niet-menselijke primaten (10e)
   ☐ Dieren in/uit het wild (10f)
   ☐ Niet gefokt voor dierproeven (11, bijlage I richtlijn)
   ☐ Zwerfdieren (10h)
   ☐ Hergebruik (1e, lid 2)
   ☐ Locatie: buiten instelling vergunninghouder (10g)
   ☐ Geen toepassing verdoving/pijnbestrijding (13)
   ☐ Dodingsmethode niet volgens bijlage IV richtlijn (13c, lid 3)

   Er is geen sprake van bijzondere categorieën van dieren, omstandigheden of behandeling van de dieren.

9. Geef aan of de dieren gehuisvest en verzorgd worden op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU. Indien niet aan deze minimale eisen kan worden voldaan, omdat het, om redenen van dierenwelzijn of diergezondheid of om wetenschappelijke redenen, noodzakelijk is hiervan af te wijken, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe.
   De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn.

10. Beoordeel of het cumulatieve ongerief als gevolg van de dierproeven voor elk dier realistisch is ingeschat en geclassificeerd. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C2)*.
    De DEC heeft zich ervan verzekerd dat de aanvrager al het mogelijke zal doen om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen.
    Het ongerief is door de onderzoekers ingeschat als matig ongerief voor het merendeel (89%) van de in totaal 7533 muizen en licht ongerief voor 11% van de dieren. De dieren ondervinden maximaal matig ongerief voornamelijk door chirurgische ingrepen en door meerdere meetsessies in wakkere dieren waarbij de kop van het dier is vastgezet. De dieren worden aan het einde van de proef gedood en het weefsel wordt ex vivo onderzocht (slice fysiologie - histologie-moleculaire samenstelling).

    Gegeven de zorgvuldige beschrijving van de procedures in de verschillende bijlagen Type Dierproeven is de DEC van mening dat het genoemde ongerief en het cumulatieve ongerief een realistische inschatting is.

11. Het uitvoeren van dierproeven zal naast het ongerief vaak gepaard gaan met aantasting van de integriteit van het dier. Beschrijf op welke wijze er sprake is van aantasting van integriteit. *(Zie Praktische handreiking ETK: Stap 1.C2). (zie bijlage I voor voorbeeld).*
    De integriteit van de dieren zal in meer of mindere mate worden aangetast tijdens de uitvoering van de proeven. De opgelegde –tijdelijke- beperking van de bewegingsvrijheid en de monoculaire deprivatie zijn de belangrijkste factoren in de aantasting van de integriteit.

12. Beoordeel of de criteria voor humane eindpunten goed zijn gedefinieerd en of goed is ingeschat welk percentage dieren naar verwachting een humaan eindpunt zal bereiken. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
    De humane eindpunten zijn duidelijk gedefinieerd. De DEC is het met de aanvrager eens dat de kans klein is dat de dieren ten gevolge van de procedures een humaan eindpunt zullen bereiken.

3V's

13. Beoordeel of de aanvrager voldoende aannemelijk heeft gemaakt dat er geen geschikte vervangingsalternatieven zijn. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
    De DEC is van mening dat de aanvrager voldoende aannemelijk heeft gemaakt dat er geen vervangingsalternatieven zijn. Om de fundamenteel wetenschappelijke kennis te vergroten over hoe verschillende types plasticiteit in het visueel systeem tot stand komen via veranderingen in feedforward en feedback verbindingen en hoe deze veranderingen zijn gereguleerd, is het noodzakelijk om proefdieren te gebruiken omdat leertaken en gedrag een belangrijke uitleesparameter zijn. Hiervoor bestaan geen alternatieven op basis van (stam)cellijnen of computermodellen.

14. Beoordeel of het aantal te gebruiken dieren realistisch is ingeschat en of er een heldere strategie is om ervoor te zorgen dat tijdens het project met zo min mogelijk dieren

wordt gewerkt waarmee een betrouwbaar resultaat kan worden verkregen. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
De DEC is van mening dat het maximale aantal van 7533 muizen te gebruiken dieren realistisch is geraamd en proportioneel is ten opzichte van de gekozen strategie en de looptijd. Er is sprake van een sequentiële gefaseerde aanpak van de subdoelen waarbij iedere volgende stap wordt afgewogen op basis van de verkregen resultaten.

15. Beoordeel of het project in overeenstemming is met de vereiste van verfijning van dierproeven en het project zodanig is opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
De DEC heeft zich ervan verzekerd dat de aanvrager al het mogelijke heeft gedaan om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen. De verwachting is dat humane eindpunten zelden zullen worden bereikt.

16. Beoordeel, indien het wettelijk vereist onderzoek betreft, of voldoende aannemelijk is gemaakt dat er geen duplicatie plaats zal vinden en of de aanvrager beschikt over voldoende expertise en informatie om tijdens de uitvoering van het project te voorkomen dat onnodige duplicatie plaatsvindt. Licht uw beoordeling toe.

    Dieren in voorraad gedood en bestemming dieren na afloop proef
    Er is geen sprake van wettelijk vereist onderzoek. Alle dieren in proef worden na afloop gedood en het weefsel wordt voor ex-vivo studies gebruikt.

17. Geef aan of dieren van beide geslachten in gelijke mate ingezet zullen worden. Indien alleen dieren van één geslacht gebruikt worden, beoordeel of de aanvrager dat in voldoende mate wetenschappelijk heeft onderbouwd. *(Zie Praktische handreiking ETK: Stap 1.C3; zie bijlage I voor voorbeeld).*
    De aanvrager gebruikt zowel mannelijke als vrouwelijke dieren behalve wanneer er vooraf redenen zijn om aan te nemen dat het gebruik van beide geslachten zal resulteren in een hoger aantal proefdieren. Dit zal altijd vooraf met de IvD worden besproken.

18. Geef aan of dieren gedood worden in kader van het project (tijdens of na afloop van de dierproef). Indien dieren gedood worden, geef aan of en waarom dit noodzakelijk is voor het behalen van de doelstellingen van het project. Indien dieren gedood worden, geef aan of er een voor de diersoort passende dodingsmethode gebruikt wordt die vermeld staat in bijlage IV van richtlijn 2010/63/EU. Zo niet, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe. Indien van toepassing, geeft ook aan of er door de aanvrager ontheffing is aangevraagd *(Zie Praktische handreiking ETK: Stap 1.C3).*
    De aanvrager geeft aan dat het noodzakelijk is om dieren te doden in het kader van het project. Het weefsel wordt voor ex-vivo studies gebruikt en de verkregen gegevens dragen bij aan het behalen van het doel. De aanvrager gebruikt voor het doden een methode die beschreven is in bijlage IV van de richtlijn en waarvoor geen aanvullende voorwaarden gelden.

    Indien niet-humane primaten, honden, katten of landbouwhuisdieren worden gedood om niet-wetenschappelijke redenen, is herplaatsing of hergebruik overwogen? Licht toe waarom dit wel/niet mogelijk is.
    Niet van toepassing.

NTS

19. Is de niet-technische samenvatting een evenwichtige weergave van het project en

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

D. Ethische afweging

1. Benoem de centrale morele vraag *(Zie Praktische handreiking ETK: Stap 3.A).*
   Rechtvaardigt het vergroten van de neurobiologische inzichten hoe verschillende types plasticiteit in het visueel systeem tot stand komen via veranderingen in feedforward en feedback verbindingen en hoe deze veranderingen zijn gereguleerd het merendeels matig ongerief dat dieren wordt aangedaan in het voorliggende project?

2. Weeg voor de verschillende belanghebbenden, zoals beschreven onder C5, de sociale en morele waarden waaraan tegemoet gekomen wordt of die juist in het geding zijn, ten opzichte van elkaar af. Om dit proces te vergemakkelijken, kunt u de belangrijkste belanghebbenden en de belangrijkste waarden die in het geding zijn waarderen. U kunt dit verwoorden in termen van gering, matig of veel/ernstig voordeel of nadeel. Geef aan waarom de DEC bevordering van waarden (baten) voor de ene belanghebbende prevaleert boven de aantasting van waarden (kosten) voor de andere belanghebbende *(Zie Praktische handreiking ETK: Stap 3.B; zie bijlage I voor voorbeelden).*
   De volgende waarden/belangen zijn in het geding (zie onderdeel C5): Waarden/belangen met betrekking tot de proefdieren: de dieren ondervinden *maximaal matig ongerief* dit wordt *door de DEC beschouwd als veel nadeel*. De belangen voor de uitvoerende onderzoekers: *veel voordeel*. De belangen met betrekking tot de doelgroepen binnen het veld van de neurobiologie: de kennisvergroting wordt door de DEC gezien als *zwaarwegend en veel voordeel*. De belangen met betrekking tot de maatschappij (patiëntengroepen): op korte termijn *gering voordeel maar op lange termijn mogelijk veel voordeel.*

3. Beantwoord de centrale morele vraag. Maak voor het beantwoorden van deze vraag gebruik van bovenstaande afweging van morele waarden. Maak daarnaast gebruik van de volgende moreel relevante feiten: belang onderzoek (C4), kennis en kunde van betrokkenen (C7), haalbaarheid doelstellingen (C8), categorieën en herkomst dieren (C9), 3V's (C14-C18), ongerief (C10-13 en C19) en relevante wet en regelgeving (C2). Onderbouw hoe al deze elementen zijn meegewogen bij de beantwoording van de centrale morele vraag, zodanig dat het navolgbaar is zonder gedetailleerde kennis te hebben van het projectvoorstel *(Zie Praktische handreiking ETK: Stap 3.C; zie bijlage I voor voorbeeld)*.
   De DEC is van mening dat de benoemde belangen van de wetenschap en samenleving in dit project zwaarder wegen dan de belangen/waarden van de proefdieren. De volgende overwegingen hebben bijgedragen tot deze conclusie:

   - Indien de doelstellingen bereikt worden, zal dit resulteren in een aanmerkelijke toename van de neurobiologische inzichten hoe verschillende types plasticiteit in het visueel systeem tot stand komen via veranderingen in feedforward en feedback verbindingen en hoe deze veranderingen zijn gereguleerd. Deze mechanismen zijn ook betrokken bij het ontstaan van ernstige ziektebeelden zoals amblyopie, autisme en verstandelijke beperking en de verkregen inzichten in neuronale plasticiteit kunnen bijdragen aan mogelijke behandelingen. Daarnaast bieden de resultaten ook een mogelijkheid om met kennis van de regulatie van plasticiteit neuroregeneratieve therapieën op te stellen.

   - De DEC is van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en

recente wetenschappelijke inzichten. De gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project.

- Het is aannemelijk dat de doelstellingen behaald zullen worden. Om dit doel te bereiken is het nodig proefdieren te gebruiken. De onderzoekers doen er echter alles aan om het lijden van de dieren te beperken waardoor het uiteindelijk ongerief van elk individueel dier, naar verwachting, beperkt blijft tot maximaal matig ongerief.

- De DEC is overtuigd van het belang van de wetenschappelijke doelstelling en het belang van de nieuwe kennis.

- De DEC is er van overtuigd dat de aanvrager voldoende kennis en kunde heeft om de doelstellingen te behalen, en tijdens de uitvoering van het project te kunnen voldoen aan de 3V-beginselen.

- De DEC is van mening dat de aanvrager bij de uitvoering van het project alle mogelijke maatregelen treft om het ongerief van de dieren te beperken en het aantal dieren tot een minimum te beperken.

Gezien bovenstaande overwegingen is de DEC van opvatting dat het bereiken van de doelstelling, op de wijze zoals beschreven in deze projectaanvraag, het gebruik van proefdieren rechtvaardigt.

E. Advies

1. Advies aan de CCD
   - ■ De DEC adviseert de vergunning te verlenen.
   - ☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
     - ☐ Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
     - ☐ Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
     - ☐ Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten: geen.
   - ☐ De DEC adviseert de vergunning niet te verlenen vanwege:
     - ☐ De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:…
     - ☐ De volgende doorslaggevende ethische bezwaren:…
     - ☐ De volgende tekortkomingen in de aanvraag:…

2. Het uitgebrachte advies kan unaniem tot stand zijn gekomen dan wel gebaseerd zijn op een meerderheidsstandpunt in de DEC. Indien gebaseerd op een meerderheidsstandpunt, specificeer het minderheidsstandpunt op het niveau van verschillende belanghebbenden en de waarden die in het geding zijn *(Zie Praktische handreiking ETK: Stap 4.A; zie bijlage I voor voorbeeld).*
   Het advies is unaniem

3. Omschrijf de knelpunten/dilemma's die naar voren zijn gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies zowel binnen als buiten de context van het project *(Zie Praktische handreiking ETK: Stap 4.B)*.
   De DEC heeft geen dilemma's gesignaleerd die binnen of buiten de context van het project vallen.

10.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen (KNAW)
[redacted]
Postbus 19121
1000 GC AMSTERDAM

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

Onze referentie
Aanvraagnummer
AVD8010020171045

Bijlagen
2

Datum 5 april 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted],

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 4 april 2017. Het gaat om uw project "Plasticity in the visual system and its regulation". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD8010020171045. Gebruik dit nummer wanneer u contact met de CCD opneemt.

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

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

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

Datum:
5 april 2017
Aanvraagnummer:
AVD8010020171045

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
5 april 2017
Aanvraagnummer:
AVD8010020171045

Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA:	80100
Naam instelling of organisatie:	Kon. Ned. Academie van Wetenschappen (KNAW)
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	54667089
Postbus:	19121
Postcode en plaats:	1000GC AMSTERDAM
IBAN:	NL33DEUT0546900054
Tenaamstelling van het rekeningnummer:	[redacted]

Gegevens verantwoordelijke onderzoeker

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

Datum:
5 april 2017
Aanvraagnummer:
AVD8010020171045

Gegevens plaatsvervangende verantwoordelijke onderzoeker

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

Over uw aanvraag

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

Over uw project

Geplande startdatum:	1 mei 2017
Geplande einddatum:	1 mei 2027
Titel project:	Plasticity in the visual system and its regulation
Titel niet-technische samenvatting:	De werking en regulatie van leren in het visuele systeem
Naam DEC:	DEC-KNAW
Postadres DEC:	[redacted]
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 1.727,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

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

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Amsterdam
Datum:	4 april 2017

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen (KNAW)
[redacted]
Postbus 19121
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info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD8010020171045

Bijlagen
2

Datum 5 april 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 5 april 2017
Vervaldatum: 5 mei 2017
Factuurnummer: 171045

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD8010020171045	€ 1.727,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL29INGB 070.500.1512 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

11.

[redacted]

Van: secretariaat DEC[redacted]
Verzonden: maandag 24 april 2017 17:17
Aan: 'info@zbo-ccd.nl'
Onderwerp: FW: AVD8010020171045 Aanvullende informatie t.a.v [redacted]

Categorieën: Dossier: [redacted]

Beste [redacted]

Ik heb vandaag namens de indieners / onderzoekers de gevraagde aanvullende informatie via NetFTP aangeleverd. De projectduur is inderdaad 5 jaar.

Misschien goed om te weten dat de KNAW [redacted] een mevrouw is..

Groet [redacted]
DEC-KNAW

From: [redacted]
Sent: woensdag 19 april 2017 9:44
To: secretariaat DEC [redacted]
Subject: FW: AVD8010020171045 Aanvullende informatie

Van: [redacted]
Verzonden: woensdag 19 april 2017 8:10
Aan: [redacted]
CC: [redacted]
Onderwerp: AVD8010020171045 Aanvullende informatie

Geachte meneer [redacted]

Op 4 april 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Plasticity in the visual system and its regulation' met aanvraagnummer AVD8010020171045. Wij hebben nog een aantal vragen over uw aanvraag. In deze e-mail leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

- U wordt verzocht informatie te verstrekken over de leeftijd van de pups die een head fixation post krijgen en de duur van de proef. Jonge pups zullen namelijk nog behoorlijk groeien. Bij langdurige bevestiging kan een head fixation post of loskomen en bloedingen veroorzaken of ongerief induceren door het blokkeren van een groeiende schedel.

-In het aanvraagformulier geeft u aan dat de gewenste duur van de vergunning 10 jaar is. Wij gaan er van uit dat dit een verschrijving is en dat u de vergunning aanvraagt voor een periode van 5 jaar. U wordt verzocht dit te bevestigen.

Opsturen binnen veertien dagen
U heeft veertien dagen de tijd om de ontbrekende informatie aan te leveren. U kunt dit aanleveren via NetFTP.

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

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,

[redacted]

Centrale Commissie Dierproeven **www.centralecommissiedierproeven.nl**

.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (Let op: nieuw e-mail adres)
De Rijksdienst voor Ondernemend Nederland (RVO.nl) stimuleert Duurzaam, Agrarisch, Innovatief en Internationaal ondernemen.

24 april 2017

AVD8010020171045.
project ‘Plasticity in the visual system and its regulation’

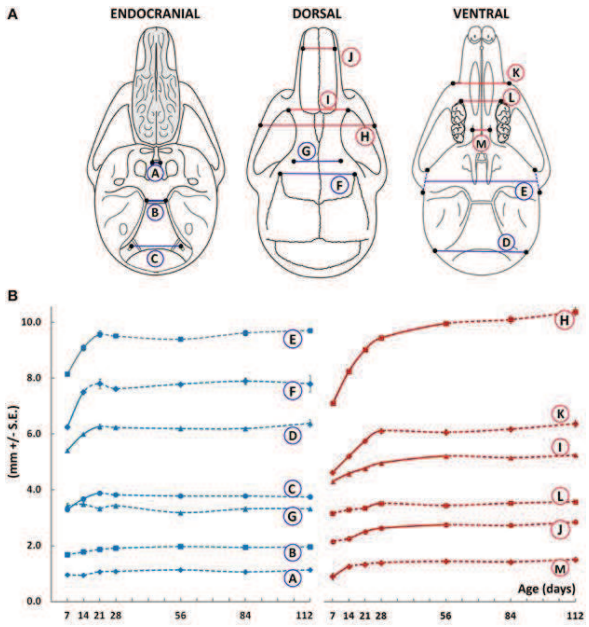
Geachte CCD,

1. De vergunning wordt inderdaad voor een periode van 5 jaar aangevraagd.

2. In antwoord op uw vraag: “U wordt verzocht informatie te verstrekken over de leeftijd van de pups die een head fixation post krijgen en de duur van de proef. Jonge pups zullen namelijk nog behoorlijk groeien. Bij langdurige bevestiging kan een head fixation post of loskomen en bloedingen veroorzaken of ongerief induceren door het blokkeren van een groeiende schedel.”, kunnen wij het volgende zeggen:

Het vastmaken van een permanente head fixation post doen wij bij muizen vanaf postnatale dag 21 (P21) of ouder. De achterkant (posterieur) van de schedel, waar we de head post plaatsen, is dan volgroeid, zoals de blauwe lijnen uit onderstaande figuur uit Vora et al. (Frontiers in Physiology, 12 January 2016) laten zien. Het vastmaken van de head fixation post bij dieren tussen P21 en P35 gebeurt voor proeven die betrekking hebben op de kritische periode voor plasticiteit in de visuele cortex. Deze proeven zullen niet langer dan 2 maanden duren. Voor experimenten in muizen na de kritische periode, dat wil zeggen ouder dan P35, kan de periode tot een jaar duren maar zal doorgaans korter zijn.

Hoewel het deel van de schedel waar de head fixation post aan vast zit dus niet meer groeit nadat de head fixation post is vastgemaakt, is het nog steeds mogelijk dat de post loslaat door andere oorzaken, zoals we in de appendices in sectie I hadden aangegeven. De meest waarschijnlijke oorzaak hiervan is slechte hechting van de lijm aan de schedel. In onze aanvraag is in de appendices al beschreven hoe we met het loslaten van de post om zullen gaan wanneer dit toch gebeurt.



Met vriendelijk groet,

[redacted]

PS. Twee leden van de IvD[redacted] hebben bovenstaande aanvullende informatie bekeken en stemmen in met de antwoorden.

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'.	80100-KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	Provide the title of the project.	Plasticity in the visual system and its regulation

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.

Brain plasticity and its regulation

The brain shows a tremendous ability to adapt to its ever-changing environment. At the root of this adaptation is the formation and refinement of neural circuits (referred to as "plasticity"), allowing our brains to develop, acquire knowledge, learn new skills and recover from injuries.

The goal of this project is to increase our fundamental scientific understanding on how plasticity is accomplished. Our hypothesis is that plasticity in the brain is brought about and modulated by several interrelated mechanisms: i) rearrangements of feedforward connections (dominant at critical periods during development, ii) rearrangements of feedback connections (dominant in adulthood), iii) changes in the influence of interneurons to temporarily enhance plasticity so that it occurs only when necessary (for example: during specific stages of development or upon punishment or reward).

Connectivity of the visual system

To reach this goal, we use the visual system of the mouse as a model. The main reason is that there is a solid understanding of the basic wiring principles of the visual system, allowing us to study where and how changes in connectivity occur during plasticity.

The visual system responds to inputs from the two eyes (Fig. 1A). These feedforward inputs from the eyes first enter the lateral geniculate nucleus of the thalamus. Because a fraction of the axons from the retina do not cross in the optic chiasm, inputs from both eyes will enter the left and right thalamus. Thalamic relay neurons project to the primary visual cortex (V1). Neurons in V1 further process the information and send projections to higher visual cortices and thalamic nuclei in which more complex visual patterns are processed. The eyes also provide input to the superior colliculus which is important for regulating, among others, eye movements. The superior colliculus, in turn, projects to the thalamus. Within the visual system there are also extensive feedback connections (Fig. 1B). Visual cortical areas receive these feedback inputs from higher visual and frontal cortices providing contextual information about the visual scene, the task that is being carried out, the state of the animal, etc. The thalamus and superior colliculus also receive feedback information from the visual cortex. This reiterative connectivity makes it possible for the brain to anticipate and rapidly interpret the flow of information that enters the brain via the eyes.

Visual responses

When visual stimuli are provided to a mouse, neurons in the visual system will respond to it. This can be recorded using electrophysiological or imaging approaches. To what properties of visual stimuli a particular neuron responds depends on the brain region it is in and the specific synaptic inputs it receives. Neurons in the thalamus, for example, mainly respond to small patches on a contrasting background while neurons in V1 respond preferentially to bars moving in a particular direction. Neurons in V1 also have a preference for inputs from the left or right eye. This property is called ocular dominance (OD). It is a direct consequence of the fact that inputs from the two eyes project to both hemispheres. Binocular vision also enables depth perception, as it allows for the comparison of images from the two eyes. Surprisingly, mouse visual cortical areas including V1 do not only respond to visual stimuli but also to motor activity, reward or punishment, anticipation, decision making, etc. This is a consequence of the feedback inputs V1 receives from higher visual and prefrontal cortices and high order thalamic nuclei. In higher visual areas, neurons respond to more complex visual properties. Here, neurons may respond to particular objects or sceneries. Taken together, the responses in the visual system are defined by the circuits that the neurons form through their synaptic connections.

Plasticity in the developing and adult visual system and its regulation

Another reason why the mouse visual system is attractive for studying plasticity is that it is relatively easy to illicit and record responses in the visual cortex and subcortical regions upon visual stimulation and to relate changes in these responses (plasticity) to connectivity changes. Of special importance for our research is that there is a well-defined critical period of development during which plasticity of OD can take place. This allows us to compare the connections that undergo plasticity during this critical period, with the connections that undergo plasticity in adulthood. Moreover, it allows us to investigate the mechanisms that regulate plasticity during the critical period, and those that regulate adult plasticity, and relate these mechanisms to the specific connections that undergo plasticity.

i) Developmental plasticity and critical periods
During development, there are defined periods during which specific brain regions show a heightened plasticity potential. Generally speaking, lower order brain regions (for example those processing sensory inputs) undergo plasticity before higher brain regions (for example those involved in executive functions).
During the critical period of OD plasticity (Fig. 2, left panel) the feedforward connections from the eyes are fine-tuned which is important for the development of normal binocular vision. If misguided, for example by dysfunction or misalignment of one eye, the visual cortex will become less responsive to this eye causing the condition known as amblyopia, or "lazy eye". When in experimental animals one of the eyes is closed during a defined period of postnatal development, V1 will become less responsive to the deprived eye while responses to the non-deprived eye are strengthened. This functional change is accompanied by (and probably caused by) extensive structural changes: thalamic axonal feedforward projections from the deprived eye retract while those of the non-deprived eye expand. Notably, OD plasticity only occurs efficiently and permanently during a critical period of development (postnatal 3-5 weeks in mice). This has made OD plasticity very informative in the study of structure/function relationships during plasticity, and in understanding the mechanisms that regulate plasticity levels – specifically the factors that regulate the onset and closure of critical periods. We have used this model extensively, to identify molecular [1-5] and cellular mechanisms underlying and regulating critical period plasticity [6,7].

ii) Adult plasticity
In the adult visual system, after critical period closure, other mechanisms underlying plasticity become dominant. In adults plasticity is induced when large reductions in sensory input occur over prolonged periods of time. For example plasticity in primary visual cortex occurs after damage to the retina. This results in the "filling in" of the visual field so that one does not experience the lesion as a black spot. This form of plasticity is important, as like OD plasticity, it is induced by visual deprivation (though requiring an almost complete loss of input from (part of) the retina), but in contrast to OD plasticity it occurs readily in adulthood. This experimental model thus allows us to address the question whether it involves rearrangement of feedforward connections like critical period plasticity, or whether it alters feedback connections, as is expected for other forms of adult plasticity. In our laboratories we have employed this form of adult plasticity to study the relationships between the gain and loss of synapses and the trafficking of mitochondria [8].
A more natural type of adult plasticity occurs in association with perceptual learning: an improvement in the ability to detect or discriminate stimuli induced by repeated practice (Fig. 2 right panel). This is the type of learning that enables the trained birdwatcher to spot rare birds in the woods that untrained people miss out on. Perceptual learning takes place in adulthood and involves various brain areas including V1. In contrast to OD plasticity, perceptual learning is strongly modulated by reinforcement signals, such as reward or punishment [9]. With perceptual learning, responses of neurons in the visual system change. Interestingly, not only responses to particular features of trained visual stimuli alter, but neurons in the visual system also start responding to anticipated reward or punishment, choices the mouse makes or task-related behaviour. In our laboratories we have established perceptual learning tasks for mice, in which mice learn to differentiate between different visual stimuli. This lets us monitor, in real time, how the responses of hundreds of neurons change during learning. A related form of reinforcement learning is visual fear learning, during which a particular visual context is associated with an aversive stimulus such as an electric shock. Once the association is made, the animal will show freezing behaviour when the visual stimulus is presented to the mouse. These plasticity paradigms typically tune responses in lower visual brain regions to more complex contextual information. Therefore we believe these forms of plasticity involve plasticity of feedback connections providing such information.
Some forms of experience-dependent plasticity that occur readily in adulthood do not require reinforcement. This is called unsupervised learning and involves, for example, reduced responsiveness to repeated stimuli that initially provide a startling or novelty response. In our hands, mice learn not be afraid of objects that unexpectedly fly over or approach quickly, a process likely to involve interaction between the visual cortex and the superior colliculus. Another example it that mice stop paying attention to objects in their cage once they become familiar with them. Unsupervised learning is thus distinct from deprivation-induced plasticity, as it is induced by visual stimuli and not by continuous lack thereof, and from perceptual learning, as it does not require reinforcement. It is therefore important to understand

whether feedforward and feedback connections between thalamus, superior colliculus and V1 rearrange during this form of plasticity, and what the regulatory mechanisms are. Moreover, unsupervised learning will also occur (unintentionally) during reinforcement learning paradigms, as the mice will get used to the handling, the experimental setup, the visual stimuli that are shown repeatedly, etc. It is therefore important to know which changes in connectivity are induced by unsupervised learning in order to isolate those that are induced specifically by reinforcement learning.

Taken together, by studying and comparing how specific feedforward and feedback connections are reorganized during critical period plasticity, adult deprivation-induced plasticity, reinforcement learning and unsupervised learning we will be able to identify overarching principles of experience-dependent connectivity changes.

iii) Regulation of plasticity

From the above it is apparent that plasticity occurs at specific developmental stages, or under particular circumstances, for example upon reward or punishment or after prolonged lack of sensory input. This means that plasticity levels must be under regulatory control allowing the different forms of plasticity, such as critical period plasticity or perceptual learning to occur only when needed. A comprehensive understanding of the fundamental mechanisms of plasticity also encompasses an understanding of these regulatory mechanisms and how they relate to the changes in connections that effectuate plasticity under different circumstances. This can be achieved particularly well in mice, as many powerful approaches for genetic modification have been developed for mice, rapidly advancing our lines of research.

We and others have identified various molecular targets that regulate plasticity levels during the critical period. These include genes that regulate axon growth and retraction, synapse maturation or the formation of the extracellular matrix. Interestingly, many of these genes strongly affect plasticity levels when their expression is modified in a specific subset of inhibitory interneurons: parvalbumin (PV)-expressing basket cells. This strongly supports the idea that these interneurons play an important role in regulating plasticity levels during critical periods [6,7]. Interneurons represent 10-20% of all neurons in the brain. In contrast to excitatory neurons that employ glutamate as a neurotransmitter, interneurons use GABA as a neurotransmitter, through which they inhibit other neurons they contact. The onset of the critical period of OD plasticity involves the development of inhibitory innervation of the cortex [10] and thalamus (Sommeijer et al, under revision for Nature Neuroscience). The further increase in the level of inhibition with development then appears to close the critical period, suggesting that a certain balance in inhibition and excitation is required for plasticity. Our work on a mouse model of Neurofibromatosis type 1 (NF1), a monogenetic developmental brain disorder associated with intellectual disability and autism, illustrates the relevance of mechanism. We find that in NF1 mice, cortical interneurons are hyperexcitable. As a consequence, these mice show early closure of the critical period. When these mice develop in an enriched environment, both cortical inhibition and critical period closure normalize. Importantly, PV+ interneurons provide important feedforward inhibition (they receive feedforward input, and inhibit excitatory neurons receiving the same inputs). Our hypothesis is that interneurons that provide feedforward inhibition are the perfect candidates to regulate plasticity of feedforward connections during critical periods.

Importantly, changes in inhibition/excitation balance also occur at the moment that plasticity is induced in adulthood. In the adult visual cortex, for example, inhibitory synapses are lost when the retina is damaged [11]. Moreover, the activity of interneurons in the visual cortex is reduced during perceptual learning [12]. These changes in inhibition appear to be crucial as activating or decreasing inhibition has been shown to reduce or increase plasticity levels [13,14]. Notably, the changes in inhibition that occur in adulthood seem to involve different subsets of interneurons than those regulating plasticity during the critical period (Fig. 2). Especially somatostatin (SST) expressing interneurons appear to be involved, which predominantly inhibit the dendrites of cortical neurons that receive feedback connections. We therefore hypothesize that interneuron subsets that gate feedback inputs are in the best place to regulate these connections in adulthood.

How the changes in the activity of inhibitory neurons or the persistence of their synapses are achieved remain unclear, as are the mechanisms through which disinhibition enhances plasticity during critical

periods or in adulthood. Our experiments have shown that during critical periods, inhibition does not have an instructive role, meaning that disinhibition does not selectively increase learned responses [7]. More likely, inhibition plays a permissive role, setting a level of plasticity. In summary, over the last years it has become clear that a temporary reduction of inhibition (disinhibition) is a critical factor in the enhancement of plasticity levels, not just during development but also in the adult visual cortex. However, this appears to involve different interneuron subsets, depending on the type of plasticity that occurs.

Open questions

Despite the growing knowledge on the anatomy of the visual system, very little is known about how experience-dependent plasticity is achieved through specific rearrangement of these connections. Most progress has been made in understanding the connectivity changes during OD plasticity during the critical period, where it is known that feedforward thalamocortical projections alter their connections to neurons in primary visual cortex. However, we have recently discovered that in contrast to what is generally believed, also within thalamus extensive OD plasticity takes place. This illustrates that even in this well-studied model, the basic principles are still not clear. What connectivity changes underlie perceptual learning, contextual fear learning or unsupervised learning in adulthood is even less well understood.

How plasticity levels are regulated is also not well understood. As explained above, disinhibition enhances plasticity. How disinhibition by specific interneuron subsets enhances plasticity, and how this can be achieved under the relevant circumstances and affect the relevant feedforward or feedback circuits needing adjustment remains unresolved. Moreover, the contribution of other mechanisms than disinhibition in the regulation of plasticity levels, such as axon growth and retraction, synapse maturation or the formation of the extracellular matrix, is unclear and requires further investigation.

The main goal of our research is therefore to gain fundamental scientific understanding on the general principles of how different types of plasticity are achieved through changes in feedforward or feedback connectivity and how these types of plasticity are regulated. We focus on the plasticity of the visual system as model.

This goal is of social relevance (see 3.3 for details). Since maladaptive plasticity mechanisms underlie a number of brain disorders (see below), understanding the overarching principles of plasticity mechanisms provides important future handles for studying the pathophysiology of such disorders. In addition, understanding the mechanisms through which plasticity levels are regulated may ultimately result in the development of approaches to enhance plasticity levels for therapeutic purposes, such as the treatment of neurodevelopmental disorders or improvement of recovery after brain damage.

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?

We aim to increase the knowledge on *the general principles of how different types of plasticity in the visual system are achieved through changes in feedforward or feedback connectivity and how these types of plasticity are regulated.*

To reach our main goal we formulated the following two sub-aims:

1. How are experience-dependent changes in brain function accomplished by rearrangements of neuronal circuits?

Our goal here is to define overarching principles of how different forms of plasticity (both developmental and adult) are achieved by modification of specific neural circuits. Our working hypothesis is that critical

periods involve rearrangement predominantly of feedforward connections, thus optimizing the circuits to process sensory inputs (Fig. 2). At later stages, associative/feedback connections are the dominant substrates of plasticity, allowing us to make novel associations. To test this hypothesis, we will identify the brain regions involved in critical period plasticity, perceptual learning, fear learning or unsupervised learning based on visual information, study the structural and functional properties of neuronal responses in these brain regions and how these change with learning, and compare the synaptic substrates of plasticity in these different developmental and adult forms of plasticity.

2. What are the cellular and molecular mechanisms that regulate plasticity levels?

Here we aim to understand how regulation of plasticity levels is achieved specifically in those brain regions and synaptic connections relevant for the learned task or function. A major focus will be on the role of disinhibition in this process. Our working hypothesis is that selective regulation of plasticity is achieved by specialized subsets of inhibitory neurons in the visual system. This hypothesis is supported by the finding that cortical (parvalbumin-expressing) interneurons that provide feedforward inhibition regulate plasticity of feedforward connections during the critical periods, while (somatostatin-expressing) interneurons that gate feedback connections regulate perceptual learning in adulthood (Fig. 2). To test this hypothesis, we will induce plasticity using defined plasticity paradigms, and compare how the activity of specific interneuron subsets and/or their synapses in the brain areas undergoing plasticity change. To establish a causal role of these changes in inhibition we will alter the activity of specific interneurons subsets or their synapses and study the effect on information processing and learning. It is also possible that specific signaling pathways are identified that regulate plasticity in defined brain regions or synaptic connections. We will therefore also use genetic or proteomic screening approaches and gene manipulation approaches to study molecular pathways regulating plasticity.

Expected outcome

We anticipate that at the end of this 5-year project we will have clearly defined whether our hypothesis that feedforward connections are the main substrate of critical period plasticity, and feedback connections are the main substrate in plasticity during adulthood is correct or not. We will also have tested whether these rules are limited to the primary visual pathway or whether they can be generalized to other (subcortical) brain regions involved in visual processing. We will also have shown which interneuron subsets regulate plasticity in thalamus and cortex during the critical period and in adulthood. We hope that we can also define roles for interneurons in other brain regions of the visual system, such as the superior colliculus. Finally, we anticipate that we will have identified molecular targets and signaling pathways that specifically affect plasticity of feedforward or feedback connections, or its regulation by specific interneuron subsets and may be relevant for understanding the pathogenesis or developing treatments for neurodevelopmental disorders such as neurofibromatosis type I.

Feasibility

The described research is highly feasible and represents ongoing and future research projects (see appendix "overview DEC proposals" for an overview of the approved DEC proposals that are currently in progress). Our laboratory is experienced and well equipped for performing the research lines. During the last 15 years we have been studying plasticity in the visual system and established a wide range of state-of-the-art experimental approaches enabling us to perform the planned research. These include experimental paradigms to induce plasticity during critical periods (OD plasticity) and in adulthood (lesion-induced plasticity, perceptual learning, habituation), approaches to record neuronal activity in vivo (single- and multiunit recordings, cell-attached recordings, optical imaging of intrinsic signal, in vivo two-photon calcium imaging) or in slices (multi-electrode patch-clamp recordings, field-potential recordings, calcium imaging), two-photon microscopy to chronically image synapse morphology in vivo, gene manipulation approaches (transgenesis, viral vectors, in utero electroporation etc.), immunohistochemistry, tissue clearing techniques, western blotting, etc. All of these techniques have resulted in publications in high impact journals [1,2,5,6,8,15-22] . Our research efforts are embedded in the environment of the NIN, providing excellent infrastructure, technological support and outstanding scientific interactions. Moreover, our research is well-linked within the national and international scientific

community.

The quality of our work is further underscored by the recognition through research funding agencies (e.g. NWO, HBP, EU). The planned research is currently funded by grants from NWO and the EU.

3.3 Relevance

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

Scientific relevance: Uncovering general principles of how neuronal networks can effectively improve their performance through experience will advance our scientific understanding on a fundamental neurobiological mechanism namely the ability of the brain to learn from experience. Our research is not only important for scientists working in the visual system, but for many scientists whose research is related to plasticity mechanisms in other brain regions. Our experiments also have impact on scientists working on artificial intelligence, brain-machine interfaces and neuroinformatics and for software developers making use of these approaches. Our laboratories perform research for the Human Brain Project, in which scientists building brain models rely on experimental information they receive from neuroscience laboratories. Moreover, the Human Brain Project incorporates the knowledge we acquire to develop robots with artificial intelligence.

Social relevance: Our research may, in the long run, have several important therapeutic implications. *First*, critical period plasticity in the visual system is an excellent model for studying the cause of amblyopia ("lazy eye") when vision is impaired during childhood due to misalignment of the optical axes or inequality of refractive power of the two eyes. Amblyopia is the most prevalent (2-4%) visual disorder in young people. Of all amblyopes, 3-18% will become visually impaired in their unaffected eye in the course of their life through injury or illness (like all people), causing binocular visual impairment and severe disability in 1:1000 people. The discovery of novel approaches to reactivate plasticity after critical period closure may result in novel avenues to treat amblyopia in adults.
Second, various neurodevelopmental disorders such as autism and intellectual disability, have been suggested to be caused by deficits in critical period plasticity. Understanding the principles of regulating plasticity levels may ultimately provide new handles on treating these diseases.

As an example: our work on mice lacking a copy of the Neurofibromatosis type 1 gene, causing autism and intellectual disability in humans, reveals that this deficit increases cortical inhibition and causes precocious critical period closure.

Third, finding approaches to (temporarily) increase cortical plasticity is also important for the development of regenerative and restorative therapeutic approaches. This is especially important for the future treatment of neurodegenerative diseases, improving brain function after physical trauma. In addition, various developments are underway for restoring vision in the blind. This includes reactivation of retinal function using optogenetics, but also implantation of electrode grids in the visual cortex to directly input visual information. These approaches will mostly likely require plastic changes to let the visual system adapt to the new types of the inputs.

The proposed experiments are primarily aimed at answering fundamental scientific questions on visual plasticity and the direct aim is not to develop new treatments. This notwithstanding, we have already found novel approaches to increase adult cortical plasticity and we are testing whether these can be applied to improve brain development in a mouse model of intellectual disability and autism (Neurofibromatosis type 1). This illustrates that our fundamental approach can lead to therapeutically relevant discoveries.

3.4 Research strategy

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

Sub-aim 1. How are experience-dependent changes in brain function accomplished by rearrangements of neuronal circuits?

This issue is addressed in different stages with decision points in between (see Fig. 3). Depending on the current knowledge on a particular form of plasticity or a particular brain region, projects may start at different stages.

A1. To determine how changes in neuronal connectivity result in functional plasticity, an appropriate paradigm is selected for inducing the form of plasticity of interest. The main categories of learning that we address and compare in the visual system are critical period plasticity (by eyelid suture) (Animal Procedures 3.4.4.1 and 3.4.4.2), lesion induced plasticity (by enucleation or retinal lesioning) (AP 3.4.4.1 and 3.4.4.2), perceptual learning (using reinforcement to train mice to respond to visual stimuli), contextual fear conditioning (by associating a visual stimulus with an aversive stimulus) and unsupervised learning (by repeatedly providing visual stimuli until the mouse is used to them) (all AP 3.4.4.3 and 3.4.4.4). The exact paradigm is selected based on the literature or our previous experience. In some cases, novel plasticity paradigms need to be developed, for example to study particular forms of perceptual learning or habituation.

A2. The next steps are aimed at determining the brain region in which the relevant information is processed (A2a) and plasticity occurs (A2b). In most cases, this is known from the literature and the brain region of study is selected together with the plasticity paradigm. However, for some types of plasticity it may be unclear where the relevant information is processed. For example, if a mouse performs a task in which it needs to learn to associate one image with a reward but not another image, one would first need to determine which visual areas are capable of differentiating between the two images. This means that if the function and anatomy of a particular brain region of the visual system is not sufficiently well-documented, we will need to investigate its function in more detail before we can examine its plasticity (AP 3.4.4.5). It may also be possible that experiments from the lab hint towards plasticity taking place elsewhere than always assumed. Our recent discovery that there is extensive OD plasticity within the thalamus illustrates this. Thus, in cases where the brain region of plasticity is not defined, recordings will need to be performed to identify and characterize regions in which a) the relevant information is processed and b) plasticity takes place. Depending on the brain region and specific plasticity type, our recordings can be done by two-photon microscopy of calcium signals, in vivo electrophysiology approaches or by optical imaging of intrinsic signal (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

Only if the relevant brain region is determined, the project is continued to study the nature of the underlying mechanisms leading to plasticity.

A3. We perform experiments to determine where in the circuitry of this brain area plasticity occurs. This analysis can take place at the anatomical or functional level. At the anatomical level this either involves chronic two-photon microscopy of fluorescently labelled neuronal structures, or post-hoc tracing experiments. At the functional level this may involve electrophysiological approaches or imaging approaches to identify the cell types/ synaptic structures that undergo plasticity. The focus will be on understanding whether changes in feedback or feedforward connections dominate and how functional and anatomical changes are related (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

A4. Finally, and if an experimental approach is available, we interfere with the observed plasticity in order to study the causal relationship (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

An example of our strategy: experiments in the lab suggested that OD plasticity may take place in thalamus. We chose to test this by establishing whether there were binocularly responsive neurons in thalamus. This was confirmed, and the nature of binocular responses was determined. Next it was decided to monocularly deprive mice during the critical period and study changes in binocular responses. We found that binocular neurons in the thalamus become more responsive to the open eye. To determine which inputs have altered (feedback, or feedforward), we plan to repeat the experiment, and measure whether the change in binocularity after deprivation remains the same when the visual cortex (which

provides feedback to thalamus) is silenced. If so, feedforward connections must be altered. Finally, to understand whether plasticity in V1 depends on plasticity in thalamus, we will inactivate plasticity genes (CamKinase II for example) in thalamus and study whether this interferes with plasticity in thalamus and V1

Sub-aim 2. What are the cellular and molecular mechanisms that regulate plasticity levels?

B1. To study how plasticity levels are regulated in a particular brain region, we first select the paradigm and brain region in which we want to address the question. This choice is made based on previous experiments (as described above) or from literature (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

B2. We will then study how cellular (inhibitory/excitatory balance for example) or molecular (gene/protein expression) properties change under these circumstances. We may study this at the anatomical level (change in synapse number) or functional level (change in synapse function or interneuron activity) or at the protein/gene expression level (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4, 3.4.4.5).

B3. Finally, we will interfere with the change in inhibition in order to understand whether there is a causal relationship between the change in inhibition and plasticity and to study the mechanisms by which inhibition regulates plasticity. This will be accomplished by manipulating the activity of specific neuronal populations (opto/chemo-genetics). Candidate genes/proteins may also be selected (from B2) and their function altered in order to test how they regulate functional properties of the relevant brain region (AP 3.4.4.5) and what their role is in plasticity (AP 3.4.4.1, 3.4.4.2, 3.4.4.3, 3.4.4.4).

Example: we knew from literature that inhibition regulates OD plasticity. We developed an approach to chronically visualize inhibitory synapses in mice. We used this approach to study the formation and loss of inhibitory synapses in V1 of mice during OD plasticity and discovered that they were rapidly lost during plasticity. To understand whether this relationship is causal, we will now interfere with the stability of inhibitory synapses by molecular intervention to study the causal relationship. We have also interfered with inhibitory interneuron function during the assessment of OD plasticity and found that changes in inhibition do not directly influence OD itself, but only its plasticity.

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.

The type of experiments we perform to address our research questions are very complex and have many experimental variables although they do not vary much in the degree of discomfort caused to the animals. It is therefore more effective to cluster the experiments based on whether plasticity is induced by visual deprivation (AP 3.4.4.1 and 3.4.4.2) or visual learning (AP 3.4.4.3 and 3.4.4.4), or whether it only involves characterization of the molecular/anatomical or functional properties of a brain region (AP 3.4.4.5) and on whether the readout is acute (AP 3.4.4.1, 3.4.4.3, 3.4.4.5) or involves chronic monitoring of neuronal function or structure and behaviour (AP 3.4.4.2, 3.4.4.4). All our experiments can be characterized by four different components (see Fig. 4):

1. Instrumentalization of the experimental animal.
2. The plasticity paradigm
3. The experimental manipulation
4. The readout

1. Instrumentalization of the experimental animal
Most of our experiments will require some type of gene modification in cells of the brain and/or cranial surgery in order to be able to use the mice for our experiments. Gene modification may be required to alter genes involved in (the regulation of) plasticity, to express proteins required for their visualization (for example by two-photon microscopy) or the modification of their function (for example by optogenetics or chemogenetics). The cranial surgery may be necessary for allowing the visualization of neurons (cranial window implant, or thinning or clearing of the skull), the implantation of a headpost for head-fixation, or the implantation of a recording chamber to allow the insertion of an electrode for

electrophysiological recordings.

Instrumentalization of the experimental animals can therefore involve the following (combination of) procedures:
1a) Breeding of wildtype, inbred and/or genetically modified mice.
1b) In utero electroporation: a limited number of neurons is genetically modified in the brain of embryos. This is achieved by opening the belly of an anesthetized pregnant female mouse, injecting DNA through the uterine wall into the brain ventricles of the embryos, and electroporating the DNA into the cells lining the ventricles. The muscle wall and skin of the belly are sutured closed again and the pups are born several days later, most of them expressing the electroporated DNA in targeted neurons of the brain.
1c) Transduction with viral vectors: a viral vector driving expression of a gene of choice is injected into the brain region to be studied, resulting in the widespread expression of the gene.
1d) Cranial surgery: for imaging experiments, a cranial window may be implanted above the brain region this is to be imaged. The skull can also be made translucent by thinning, or by applying nail polish. In both cases, a head post is attached to the skull allowing fixation of the head. For electrophysiology experiments, a head post and a recording chamber or electrode may be inserted allowing head-fixation and performing recordings.

Combinations of the different genetically modified mouse models are common. In many cases, viral vectors are used that are cre-dependent. These are injected in transgenic mice expressing cre in a particular brain region or cell type. Another example can be the use of in utero electroporation to express a receptor for a virus in a limit number of neurons, followed by transduction using a viral vector later in life that will selectively infect these neurons allowing the tracing of the synaptic inputs these cells receive.

2. Plasticity paradigms:
As explained above, different plasticity paradigms are used.

Visual deprivation paradigms (3.4.4.1 & 3.4.4.2):
2a) OD plasticity is induced by suturing one eyelid closed under anaesthesia. The eye remains closed for several days and is reopened before assessment of visual responses (see below).
2b) Retinal lesions can made in adult mice. This either involves binocular lesions in the binocular-projecting part of the retinas, or a monocular lesion in the monocularly projecting region (in order to eliminate all retinal input to the cortex responding to a particular region in the visual field). The lesion is made under anesthesia using a powerful laser. Lesion-induced plasticity can also be induced by enucleation under anesthesia.

Visual learning paradigms (3.4.4.3 & 3.4.4.4):
2c) Perceptual learning involves the training of mice in a particular task. The exact task will vary depending on the exact question and experimental readout (see below). In head-fixed tasks, mice first habituate to being head-fixed and in some cases, to walk on a treadmill without being fearful. Then they learn to associate a particular visual stimulus presented to them with a behavioural response (for example, lick left if a square is shown, lick right if a circle is shown). This training involves reinforcement, i.e. reward or in some cases, punishment. Reward usually consists of providing milk through a lickspout. Mice in such paradigms are water-restricted and get most of their fluid intake during the training. Head-fixation is required for many types of imaging or electrophysiology (see below). Head-fixation is not always necessary. In such cases, training may take place in a home cage or specialized environment.
2d) Visual fear learning involves pairing a particular visual context with an aversive stimulus. Upon conditioning, the mouse will show freezing behaviour upon seeing the conditioned visual stimulus.
2e) Unsupervised learning lets mice get used to visual stimuli that are novel or naturally perceived as threatening. A threatening stimulus can be a disc increasing in size projected on the top of the cage or a fly-over stimulus. These stimuli resemble approaching predators.
In many cases the paradigms will be carried out while the mice are head-fixed, allowing us to monitor or manipulate neuronal activity or morphology (see under 4) while plasticity is being induced.

3. The experimental manipulation
In order to causally test the involvement of a particular cell type or protein in the processing of relevant information or (the regulation of) its plasticity we use several approaches to interfere with the function of specific neurons or proteins.
To achieve this we use the following tools:
3a) Focal modulation:
We will locally stimulate or inhibit neural activity using transcranial, local and intracellular electrical stimulation and inactivation, and focal ultrasound delivery.
3b) Activation of designer proteins
We will activate designer proteins that have been expressed in the instrumentalization phase. We use different types of such proteins:
Optogenetics: after expressing light-sensitive proteins in specific neuronal subsets, their activity can be increased, decreased or more selectively modulated by shining light on them.
Chemogenetics: after expressing receptor proteins that bind a ligand that has little or no biological effect on other cells in the body, the ligand can be administered to the mouse resulting in the activation, suppression or more selective modification of neuronal function.
Ultrasound-mediated neuronal modulation: novel approaches for modifying neuronal activity are developing rapidly. One such development is the expression of mecanoreceptors that modify neuronal activity in an ultrasound -manner.
3c) (Opto)pharmacology: Pharmacology will be applied to alter neuronal activity or to target specific receptors. In some instances, Optopharmacology will be used (these are pharmacological agents that are activated by light).
3d) Housing conditions: By changing the conditions under which the mice are housed, plasticity levels can be manipulated. Environmental enrichment is known to alter the inhibition/excitation balance and to affect plasticity (this is illustrated by our finding that environmental enrichment reduces inhibition in NF1 mice and normalizes critical period closure in these animals). It has also been shown that rearing mice in complete darkness can keep the critical period for OD plasticity open. Housing adult mice in complete darkness can reopen the critical period.

4. The readout
The readout of neuronal activity in a particular brain region can be measured by various approaches. Each approach has its strengths and weaknesses, and which approach is used depends on the exact question and the brain region.

Acute assessment of molecular or neuronal properties using electrophysiology, histology or molecular analysis (3.4.4.1, 3.4.4.3, 3.4.4.5):
4a) In vivo probe recordings: this involves the introduction of an (multi-array) electrode into brain of the mouse and record electric activity of neurons, or an electrochemical probe for voltammetry. Before or during the recording session, the mouse is anesthetized, a hole is drilled in the skull and the electrode is inserted (see under 1d). Acute recordings are performed in awake or anesthetized mice and the animal is sacrificed immediately after completion of the recordings.
4b) Acute imaging of intrinsic signal or fluorescent indicators of neuronal activity. Optical imaging of intrinsic signal involves measuring the reflectance of red light shone on the brain. Changes in blood oxygenation and hemodynamics due to neuronal activity alter the light reflectance. For acute imaging, the scalp of the mouse is removed under anesthesia and the mouse is placed in the imaging setup and recordings take place for several hours while visual stimuli are presented.
Two-photon microscopy of neuronal activity: in these experiments two-photon microscopy is employed to detect changes in neuronal activity. To be able to detect neuronal morphology or activity, a fluorescent reporter is required. This can be a chemical reporter, which needs to be loaded into the brain before the onset of the recording. This is achieved by injection of the dye into the brain using a pipet while the mouse is under anesthesia. In most cases, however, genetically encoded reporters are used based on fluorescent proteins. Expression of these proteins thus requires gene transfection using viral vectors or in utero electroporation, and/or the use of transgenic mice. These procedures have to take place well before the measurement takes place. The recordings generally take place in head-fixed mice (see above), under anesthesia or in awake mice in which a cranial window has been implanted. A head stage is also implanted for fixating the head under the microscope or wide field imaging setup. Visual

responses are elicited by presenting visual stimuli to the mouse. Two-photon microscopy of calcium signals allows measuring the activity of hundreds to thousands of individual neurons in real time.
Wide field imaging of fluorescent markers for neuronal activity makes use of the same principles as two-photon microscopy, but at a different scale. This approach involves macroscopy and does not allow activity measurements at the single cell level, but at the population level. The advantage is that imaging of a much larger part of the cerebral cortex of the mouse can be imaged. Also for widefield imaging, the skull needs to be made translucent, either by the implantation of a cranial window or by application of agents rendering the skull translucent.
4c) Slice electrophysiology: mice are anesthetized and sacrificed, and the brain is rapidly removed, cooled and sliced. The brain slices can then be used to record neuronal/synaptic activity by electrophysiology or to acutely induce plasticity by electrical stimulation.
4d) Tissue extraction: to isolated RNA, DNA or proteins from tissue the mice are anesthetized and sacrificed, and the tissue is removed. In most cases the tissue is then snap frozen and stored for later extraction of the required molecular components.
4e) Histology: to collect tissue for histology, the mice are anesthetized and perfused with fixative. The tissue is then removed and post-fixed after which it is sliced and stained using immunohistochemistry. This approach is essential for anatomical and molecular assessment of brain tissue.
In many cases, combinations of the above will be used, such as histology after an in vivo electrophysiology experiment.

Chronic recording of neuronal activity and morphology (3.4.4.2, 3.4.4.4).
4f) In vivo probe recordings: (see 4a). Mice undergoing chronic recordings, have been implanted with a recording chamber or electrode (see 1d). The electrode may either be implanted chronically, or, alternatively, a recording chamber is implanted and the electrode is inserted only during the recording after which the whole is closed with bone wax. In both cases, the recording can be done repeatedly in awake, behaving mice or in anesthetized mice. This read-out may be followed by a final (acute) readout session described under 4a through 4e.
4g) Optical imaging of intrinsic signal (see above) can also be performed chronically. In this case skull clearing or cranial window implantation is performed as described above and a head-post needs to be implanted.
Two-photon microscopy of neuronal structure and/or neuronal activity: in these experiments two-photon microscopy is employed in the same way as described in 4b. However, the recording is performed repeatedly in awake or anesthetized animals, allowing us to detect changes in neuronal morphology (such as synapse size or density) or neuronal activity over prolonged periods of time. A cranial window and head-post is inserted as described under 1d. In case repeated imaging in deep structures of the brain is required, a guide tube for a GRIN lens is implanted in the brain, allowing chronic and repeated insertion of the lens.
Finally, wide field imaging of fluorescent markers for neuronal activity (see above) can also be performed chronically, in which case a head-post needs to be implanted. This read-out may be followed by a final (acute) readout session described under 4a through 4e.

Again, combinations of these techniques are often used: for example optical imaging of intrinsic signal to determine the retinotopy of V1 (i.e. how the visual field is processed within V1) followed by two-photon microscopy of fluorescently labelled neurons, or electrophysiological recordings of neurons that are fluorescently labelled for chronic imaging.

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.

This project includes all steps from developing plasticity paradigms, to identifying the relevant brain regions and neural substrates mediating and/or regulating learning, the testing of causal relationships, and investigating errors in neurodevelopmental disorders as described above.

The first subaim (Fig. 3A) is to compare how different forms of plasticity are mediated by changes in neural connectivity. In many cases, we will make use of established plasticity paradigms, such as OD

plasticity or lesion-induced plasticity. When we study circuit changes involved in altered behavior, we will adapt existing behavioral paradigms for perceptual learning, contextual fear conditioning or habituation in order to make them suitable for the automated tracking of behavior in combination with in vivo electrophysiology or imaging approaches. Once we achieve this for a particular form of visual plasticity we next identify the brain regions processing the relevant information. If identified (go/no-go) we will establish whether plasticity occurs in this brain region before continuing with detailed analyses (go/no-go). We then determine where in the circuitry of this brain region plasticity occurs, which will happen at both the anatomical and functional level by observing and testing where changes in connectivity or function occur. Finally, we interfere with the observed plasticity in order to study the causal relationship.

The second subaim (Fig. 3B) is to understand the cellular and molecular mechanisms that regulate plasticity levels. We will select a particular brain region and plasticity paradigm for which we want to address this question based on our previous results, obtained in subaim 3a, and the literature. We will assess changes in cellular or molecular properties during plasticity induction that are linked to plasticity regulation. Identified targets will be experimentally modified to validate a causal relationship and understand the underlying mechanisms.

It is important to note that there is overlap between the animal studies described in this project and those in earlier DEC-approved protocols and on which we are performing currently our animal experiments. After a license for this project has been obtained, all experiments will formally be executed under this new license. To illustrate the scientific topics we are working on, we included with this application a document entitled "*Overview current DEC protocols*". This also demonstrates that both the proposed Animal Procedures together play an essential role in obtaining our research objectives.

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

Serial number	Type of animal procedure
1	**Readout after visual deprivation:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual deprivation.
2	**Readout during visual deprivation:** Chronic in vivo assessment of neuronal function or anatomy in control mice and mice during plasticity induction by visual deprivation.
3	**Readout after visual learning:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual learning
4	**Readout during visual learning:** Chronic in vivo assessment of neuronal function or anatomy in control mice and mice undergoing plasticity induction by visual learning
5	**Readout in naïve mouse:** Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in mice in without plasticity induction
6	
7	
8	
9	
10	

15.

Centrale Commissie Dierproeven

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Aanvraagnummer
AVD8010020171045
Bijlagen
1

26 APR. 2017

Datum 25 april 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 4 april 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Plasticity in the visual system and its regulation" met aanvraagnummer AVD8010020171045. Wij hebben uw aanvraag beoordeeld.

Op 24 april 2017 heeft u uw aanvraag aangevuld. Op 19 april 2017 hebben wij u om aanvullende informatie gevraagd over de head fixation post en de looptijd van de vergunning. Wij kunnen ons vinden in uw toelichting.

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.
Met het oog op artikel 10a, lid 1, zijn er algemene voorwaarden gesteld.

In uw aanvraag geeft u aan dat er sprake is van overlap tussen lopende DEC protocollen en de huidige vergunningsaanvraag. Om te borgen dat er geen sprake zal zijn van overlap, is een voorwaarde toegevoegd aan deze vergunning.

In uw aanvraag geeft u aan in principe zowel mannelijke als vrouwelijke dieren te gebruiken. Er worden namelijk geen verschillen verwacht tussen mannelijke en vrouwelijke. Wel gaat u data verzamelen over beide geslachten. Mocht blijken dat er toch verschillen zijn, wilt u de desbetreffende proeven alsnog met of mannelijke of vrouwelijke dieren uit kunnen voeren. Wij hebben hier geen bezwaar tegen. Wij vinden het wel van belang dat deze

afweging in afstemming met de IvD wordt gemaakt. Om dit te borgen is er een voorwaarde aan de vergunning toegevoegd.

U kunt met uw project "Plasticity in the visual system and its regulation" starten. De vergunning wordt afgegeven van 1 mei 2017 tot en met 30 april 2022. Deze termijn is anders dan in uw aanvraag, omdat een vergunning maar voor maximaal 5 jaar mag worden afgegeven.

Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-KNAW gevoegd. Dit advies is opgesteld op 4 april 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
In aanvulling op het DEC-advies stelt de CCD voorwaarden. De voorwaarden staan in de vergunning beschreven. Voor het overige nemen wij het advies van de DEC over, inclusief de daaraan ten grondslag liggende motivering. Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

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

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

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

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

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

Centrale Commissie Dierproeven

namens deze:

[redacted]

Algemeen Secretaris

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

Datum:
25 april 2017
Aanvraagnummer:
AVD8010020171045

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Kon. Ned. Academie van Wetenschappen (KNAW)
Adres:	Postbus 19121
Postcode en plaats:	1000GC AMSTERDAM
Deelnemersnummer:	80100

deze projectvergunning voor het tijdvak 1 mei 2017 tot en met 30 april 2022, voor het project "Plasticity in the visual system and its regulation" met aanvraagnummer AVD8010020171045, volgens advies van Dierexperimentencommissie DEC-KNAW. Hierbij is afgeweken van het DEC-advies. Er worden aanvullende voorwaarde(n) gesteld. Zie samenvatting
De functie van de verantwoordelijk onderzoeker is Groepsleider & Hoogleraar.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 4 april 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 24 april 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 4 april 2017;
c Advies van dierexperimentencommissie d.d. 4 april 2017, ontvangen op 4 april 2017.
d De aanvullingen op uw aanvraag, ontvangen op 24 april 2017

Aanvraagnummer:
AVD8010020171045

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1. Readout after visual deprivation: acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual deprivation.				
	Muizen (Mus musculus) / Moeders: 83, Embryos (>E11): 250, overig: 1000	1.333	100% Matig	
3.4.4.2. Readout during visual deprivation: Chronic in vivo assessment of neuronal function or anatomy in control mice and mice during plasticity induction by visual deprivation.				
	Muizen (Mus musculus) / Moeders: 50, Embryo's: 150, overig: 600	800	100% Matig	
3.4.4.3. Readout after visual learning: Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in control mice and mice after plasticity induction by visual learning.				
	Muizen (Mus musculus) / Moeders: 83, Embryo's (>E11): 250, overig: 1000	1.333	80% Matig 20% Licht	
3.4.4.4. Readout during visual learning: Chronic in vivo assessment of neuronal function or anatomy in control mice and mice undergoing plasticity induction by visual learning.				
	Muizen (Mus musculus) / Moeders: 50, Embryo's (>E11): 150, overig: 600	800	100% Matig	
3.4.4.5. Readout in naïve mouse: Acute in vivo or ex vivo assessment of neuronal function or anatomical/molecular make-up in mice without plasticity induction.				

Aanvraagnummer:
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	Muizen (Mus musculus) / Moeders: 167, Embryo's (>E11):500, overig: 2600	3.267	82% Matig 18% Licht	

Voorwaarden

Op grond van artikel 10a1 lid 2 van de Wet op de dierproeven zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat go/no go momenten worden afgestemd met de IvD.

In artikel 10, lid 1 sub a 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 afstemming met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarde wijzigen of intrekken.

Daar waar er sprake is van overlap tussen de in deze vergunning vergunde dierproeven en eerder goedgekeurde DEC protocollen zullen de dieren en experimenten na het verlenen van de vergunning formeel onder deze vergunning gaan vallen, zoals u in uw aanvraag ook heeft aangegeven. Hierdoor is er geen sprake meer van overlap.

Mannelijke en vrouwelijke dieren moeten in principe in evenredige aantallen gebruikt worden. Indien gedurende het project blijkt dat er in specifieke situaties geslachts-specifieke effecten zijn, kunt u met de IvD afstemmen of het noodzakelijk is voor het behalen van de doelstelling om 1 geslacht te gebruiken.

Aanvraagnummer:
AVD8010020171045

Weergave wet- en regelgeving

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

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

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

kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

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