Instantie voor Dierenwelzijn Utrecht

	Dossier: AVD1080020173404	
		Aanwezig
1	NTS	X
2	Aanvraagformulier	X
3	Projectvoorstel	X
4	Bijlage beschrijving dierproeven	X
5	DEC-advies	X
6	Ontvangstbevestiging	X
6.1	Evt. Vragen CCD aan aanvrager	X
6.2	Evt. antwoorden aanvrager	X
7	Beschikking en vergunning	X
8		
9		
10		

Centrale Commissie Dierproeven

Format
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Het ontrafelen van moleculaire mechanismen die aan de basis van de ontwikkeling en functie van zenuwcellen staan
1.2	Looptijd van het project	Looptijd 5 jaar
1.3	Trefwoorden (maximaal 5)	Zenuwcellen, centraal zenuwstelsel, hersenontwikkeling, hersenziekten

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☒ Fundamenteel onderzoek
- ☐ Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1 Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)

Onze hersenen en ons ruggenmerg, samen aangeduid als het centrale zenuwstelsel, bevatten miljarden hersencellen. Hersencellen vormen gespecialiseerde structuren waarmee ze onder andere in contact staan met andere hersencellen. Door middel van deze structuren geven ze signalen aan elkaar door. Het transport van bouwstenen en signaalstoffen is essentieel voor het maken van deze structuren en het overbrengen van signalen. Hersencellen blijven zich tijdens hun volledige levensduur aanpassen om hun vele complexe functies uit te kunnen voeren en om te overleven.

De ontwikkeling en werking van hersencellen wordt op moleculair niveau

		bepaald. Verstoringen op moleculair niveau kunnen de ontwikkeling en werking van hersencellen op een specifieke manier beïnvloeden, wat kan leiden tot een neurologische aandoening. Door te begrijpen welke moleculaire processen ten grondslag liggen aan de complexe werking van hersencellen, en in het bijzonder het transport binnen de cel, komen we meer te weten over hun functioneren in zowel het gezonde als het zieke zenuwstelsel. Deze kennis legt de basis voor het verkennen van nieuwe manieren om neurologische aandoeningen te behandelen.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Gebaseerd op ons vorige werk, onze specialistische kennis en beschikbare infrastructuur zijn er een aantal specifieke doelstellingen. Zo willen we bepalen welke moleculen verantwoordelijk zijn voor het transport binnen hersencellen. Ook willen we bepaalde transportmoleculen kunstmatig laten binden aan hun moleculaire vrachten, om het vrachtverkeer naar contactpunten tussen hersencellen (zogenaamde 'synapsen') te reguleren en transportdefecten in zieke hersencellen te herstellen. We zullen moleculen karakteriseren die de afgifte van moleculaire vrachten bij synapsen reguleren en kijken hoe zij bijdragen aan de sterkte van de synaps, omdat de sterkte van de synaps mede bepaald wordt door de aanlevering van bouwstenen en signaalstoffen. Daarnaast willen we de relatie tussen gespecialiseerde, hersencel-specifieke structuren en transport onderzoeken in gezonde en zieke hersencellen. Tot slot evalueren we wat er precies mis is met bepaalde zieke hersencellen, en willen we cellulaire mechanismen identificeren die mogelijk betrokken zijn bij misvorming van het brein als gevolg van mutaties in specifieke genen. Deze inspanningen zullen leiden tot beter begrip van het functioneren en de ontwikkeling van hersencellen, wat als basis dient voor het verkennen van nieuwe therapeutische mogelijkheden voor neurologische aandoeningen.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Naar schatting zijn maximaal 6500 ratten en maximaal 4715 muizen nodig. Hiervan is minder dan 10% van de ratten volwassen, en zijn maximaal 1115 van de muizen volwassen. De rest is embryonaal.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	We verwachten geen negatieve gevolgen voor het welzijn van de dieren tot ze worden gedood, ten behoeve van de isolatie van weefsel voor ons onderzoek. Het doden is het enige ongerief voor de dieren, wordt door getrainde onderzoekers uitgevoerd en veroorzaakt geen pijn voor de dieren.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	We verwachten dat de dieren licht ongerief ervaren tijdens de handeling waarbij ze worden gedood.
3.6	Wat is de bestemming van de dieren na afloop?	De dierproef in dit onderzoek bestaat uit het isoleren van zenuwweefsel nadat de dieren gedood zijn.

4 Drie V's

4.1 Vervanging

Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.

Het onderzoek wordt verricht op hersencellen. Hersencellen zijn niet-delende cellen. Dit houdt in dat ze niet vermeerderd kunnen worden. Daarom moeten er herhaaldelijk nieuwe hersencellen geïsoleerd worden uit dieren.

Omdat de omgeving van de hersencel belangrijk is voor zijn functioneren, bestuderen we de rol van de geselecteerde moleculen ook in de context van weefsels. Momenteel kunnen we een soortgelijke situatie niet nabootsen in een kweekschaal. Daarom zijn we gebonden aan het gebruik van proefdieren.

Vervangende technieken zijn momenteel in ontwikkeling, maar hebben zich nog onvoldoende bewezen om de komende vijf jaar bij te kunnen dragen aan betrouwbare onderzoeksresultaten.

4.2 Vermindering

Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.

Onze onderzoeksplannen zijn gebaseerd op uitgebreid vooronderzoek; indien mogelijk worden verkennende experimenten uitgevoerd in niet-zenuwcellijnen voordat de overstap naar gekweekte hersencellen wordt gemaakt. Vervolgens maken we gebruik van kleinschalige 'pilot' experimenten om onze methoden te optimaliseren en te berekenen hoeveel dierlijk materiaal minimaal nodig is om met onze experimenten tot wetenschappelijk verantwoorde conclusies te komen.

Als blijkt dat onze kandidaat-moleculen een belangrijke, nog onbekende rol spelen in de ontwikkeling en werking van hersencellen, maken we de overstap naar volledige breinstructuren. Hierbij maken we gebruik van de nieuwste technieken en hoogwaardig materiaal en gereedschap om de hoeveelheid proefdieren te minimaliseren.

Tenslotte stemmen we al onze dierproeven af binnen onze onderzoeksgroep en met andere groepen op de campus, zodat de weefsels van een dier optimaal worden gebruikt.

4.3 Verfijning

Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.

Ratten en muizen lijken qua ontwikkeling van hun zenuwstelsel sterk op de mens. Dit maakt hun hersencellen en weefsels uitermate geschikt om fundamentele moleculaire processen in zenuwstelsels te bestuderen, die in veel gevallen te vertalen zijn naar het functioneren van zowel gezonde als zieke zenuwstelsels in de mens. We hebben bovendien veel ervaring met proeven met ratten en muizen, waardoor we efficiënt kunnen werken.

Voor de verdere verfijning van onze dierproeven gebruiken we gekweekte hersencellen van ratten, omdat deze een hogere opbrengst hebben per dier dan

	muizen. In het geval van weefsels gebruiken we muizen, omdat dit de internationale standaard is, waardoor ons onderzoek beter te vergelijken is.
Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	De dierproef, dus het doden, wordt uitgevoerd door bevoegd, ervaren en competent personeel volgens de wettelijke richtlijnen.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

Centrale Commissie Dierproeven

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of 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* 10800
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	Universiteit Utrecht
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	30275924
Straat en huisnummer	Instantie voor Dierenwelzijn Utrecht

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.

Postbus	12007
Postcode en plaats	3501AA Utrecht
IBAN	NL27INGB0000425267
Tenaamstelling van het rekeningnummer	Universiteit Utrecht

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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

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

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☐ 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	1 - 1 - 2018
		Einddatum	1 - 1 - 2023
3.2	Wat is de titel van het project?	Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system	
3.3	Wat is de titel van de niet-technische samenvatting?	Het ontrafelen van moleculaire mechanismen aan de basis van de ontwikkeling en functie van zenuwcellen	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC Utrecht
		Postadres	Postbus 85500 3508 GA Utrecht
		E-mailadres	dec-utrecht@umcutrecht.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1035 Lege

☐ Wijziging € Lege

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

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☐

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). De ondergetekende verklaart:

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

Naam [redacted]

Functie

Plaats Utrecht

Datum 18-09-2017

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'.	10800
1.2	Provide the name of the licenced establishment.	University Utrecht
1.3	Provide the title of the project.	Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system

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.

Our brain and spinal cord, referred to as the central nervous system (CNS), contain billions of nerve cells (neurons). Each of these neurons generates a long nerve fiber (the axon) that connects to other neurons or other cells outside of the CNS. Most axons are organized in large bundles that transmit information between different brain regions or to other organs and are crucial for the regulation of functions ranging

from muscle contraction to complex cognitive tasks. The neurons in our central and peripheral nervous system (CNS and PNS) are highly polarized cells, specialized in the processing and transmission of electrical signals. The neurons in the CNS normally generate one signal-transmitting axon that connects to other neurons or cells outside the CNS, and several dendrites, which receive the information from other neurons. The connections between nerve cells where signals are transmitted from one cell to the other are incredibly small (< 1µm), but highly complex and dynamic structures, called synapses. Most axons, dendrites and synapses are formed during embryonic and early postnatal development, but are dynamic structures which can change, be maintained or degenerate during life. Neurons use active transport driven by molecular motor proteins to move cargo into axons and dendrites to their specific sites of action. These transport mechanisms are crucial for synapse development and function.

Abnormalities in intracellular transport are thought to be a critical factor in the development and degeneration of neurons in both the CNS and PNS. Cytoskeleton remodeling and dysfunctional axonal transport are emerging as some of the earliest and critical pathogenic events in well-known neurodegenerative diseases like Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and spinal muscular atrophy (SMA) (De Vos et al., 2008; Goldstein, 2012; Morfini et al., 2009). Disruption of axonal microtubules and/or transport can result in vesicle-trafficking impairments, alter specific cargo interactions and cause defects in retrograde survival signals, ultimately leading to neuronal death and loss of brain function (Coleman, 2011; Perlson et al., 2010; Saxena and Caroni, 2007). AD is only one example of a neuronal disease linked to defects in cytoskeleton organization and transport, but illustrates the importance of these processes very well: axonal pathologies, including abnormal accumulations of proteins and organelles have been observed in AD patients, and can severely hinder neuronal transport pathways (Gunawardena and Goldstein, 2005; Millecamps and Julien, 2013). For example, microtubule-associated protein Tau forms neurofibrillary tangles (NFTs) when hyperphosphorylated. These tangles are aggregates and one of the pathological hallmarks of AD. Importantly, Tau binds to a major axonal motor protein complex (Dixit et al., 2008) and AD disease-associated mutations affect axonal trafficking (Zhang et al., 2004). The predominating view is that pathological changes in Tau result in Tau aggregation, causing disruptions in microtubule-based transport. In addition, defects in cytoskeleton-based transport, docking and fusion of vesicles with neurotransmitters with the plasma membrane at the synapse are early events in the disease (Kaeser et al., 2009; Liu et al., 2014; Sudhof, 2012).

Several recent studies describe that disruptive mutations in microtubule motor proteins and their regulating binding proteins in human are directly linked to human motor neuropathies (e.g. SMA) and axonal CMT (Charcot-Marie-Tooth) disease and/ or malformations of cortical development, including lissencephaly, pachygyria and polymicrogyria, pointing to the essential function of motor proteins and their regulators in nerve cells in health and disease.

In fact, there is a growing body of evidence that multiple well-known neurodevelopmental and neurodegenerative diseases, but also an increasing number of less described neurological diseases and neuronal malformations/ malfunctions in humans are linked to defects in actin- and microtubule-dependent transport and regulation. Despite that, we still lack the mechanistic understanding of cytoskeleton organization and axonal vesicle transport in general and how axonal transport is affected in disease or gene loss-of-function situations. This lack of understanding of these cellular mechanisms is one of the main reasons why an effective treatment for many of these malformations/ malfunctions and/ or diseases is still missing. For example, worldwide there are millions of people suffering from neurodegenerative disorders, like AD, ALS and SMA and in the absence of effective treatment, this presents a staggering health-care burden expected to triple by 2050 in patient numbers and costs for society. Current available therapies for adult onset neurodegenerative diseases provide symptomatic relief but do not significantly modify disease progression nor cure the disease. Therefore, to be more successful in designing therapy, alternative strategies have to be found that interfere earlier with disease progression. To achieve this, better mechanistic insight into the general cellular and molecular processes and the earliest phases of the disease are needed.

Our overall aim is to understand cytoskeleton and transport processes and to identify the key molecular players in these processes in **general** (healthy neurons), as well as in "diseased"* neurons/ neurons with modified gene expression to generate loss of function (LOF) or gain of function (GOF) situations for

specific genes. These findings will help to provide a better understanding of the role of these processes in neuronal development, maintenance and degeneration and can provide a basis for the development of effective treatment strategies in the future. We will focus thereby on general molecular mechanisms in healthy neurons which will also help to understand the mechanisms in diseased neurons, where these mechanisms are altered. Moreover, we will specifically analyze these processes in diseased neurons, where a specific gene is mutated to better understand the mechanistic connections between disease phenotypes and cell-molecular cytoskeleton and transport based mechanisms.
Our laboratory contributed already in the past to the recent advances in the field [redacted]. For example, we recently developed [redacted] and identified cytoskeletal-related proteins that are part of the neuronal transport machinery [redacted]. This puts us in a unique position to investigate fundamental mechanisms of axonal cargo transport defects in neurodevelopmental and neurodegenerative diseases.
We now aim to further investigate the special transport mechanisms, regulation of cytoskeleton dynamics and synapse organization. Understanding the molecular mechanisms underlying these processes in healthy and diseased neurons will significantly contribute to our understanding of neurodevelopmental and neurodegenerative diseases and malformations/ malfunctions and may provide new strategies for intervention and treatment.

*We define "diseased" neuron here as a neuronal cell which shows an abnormal phenotype. In most cases this occurs due to a genetic mutation, (potentially) linked to human malformations/ malfunction/ neurological diseases, resulting loss-of-function or gain-of-function situation.

3.2 Purpose

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

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

The overall goal of this project is to understand the regulatory mechanisms of cytoskeleton dynamics, cytoskeleton-based transport processes and transport-dependent synapse organization in healthy and disease neurons. These new findings will help to understand the role of these processes in neuronal development, maintenance and degeneration and to understand the pathogenic mechanisms involved in neurodevelopmental and neurodegenerative disease at the molecular, cellular and systems levels. This information can provide a basis for the development of effective therapeutic strategies for these disorders.
Based on our previous work, know-how, and available infrastructure the specific aims/ research objectives of this project are to:

1. Determine which motor proteins regulate cargo transport and engineer inducible motor protein-cargo complexes to spatially control vesicle trafficking to synapses and override transport defects in diseased/ LOF neurons.
2. Characterize the molecules that control cargo delivery at synapses and that affect the relation between synapse strength and synaptic cargo trafficking.
3. Investigate specialized, neuron-specific cell structures such as the axon initial segment (AIS) and their effect and function on cargo transport and sorting in axons in healthy and diseased/ LOF neurons ([redacted]
4. Evaluation of specific diseased neuron/ LOF and GOF phenotypes, and identification of the cellular mechanisms implied in the neuronal malformation/ malfunction which are associated with mutation in specific genes ([redacted] ko mouse as model system for SMA, in the [redacted] as model system the cortical malformations associated with mutations in [redacted] in humans and in the [redacted] ko mouse as model system for [redacted]).

These specific aims are a continuation of work currently ongoing in the lab. This includes the identification of intracellular proteins, the characterization of recently identified intra- and extracellular molecules, the nano-spatial analysis of the cytoskeleton and the synapse in primary cultured rodent hippocampal and cortical neurons and studying the role of these molecules in neuronal development and neuronal migration *ex* and *in vivo*.

In specific, we will follow a clear strategy (described in 3.4) and aim to determine/ discover in the coming five years specific motor proteins and molecules that regulate transport and cargo delivery by screening putative candidate molecules in knockdown screens in primary cultured rodent neurons (*in vitro*); to characterize the molecular function of these molecules and to investigate the role of specialized structures of the neuron and cytoskeleton organization by identifying and characterizing the molecules involved in the establishment and maintenance of these structures *in vitro* by generating LOF and GOF situations. Together, we aim to screen about 500 candidate molecules and to identify and characterize about 40 molecules of key importance for these purposes. For 25 of them, we also aim to investigate their cellular function in brain tissue cultures *ex vivo***. Following this strategy, we already discovered several molecules of key importance in the past. 6 of these molecules we selected because mutations in these genes have been shown/ suggested to be associated with malformation/ malfunction in the CNS and PNS of humans and already generated knockout mouse models. In the period of this project, we will now investigate the role of these 6 molecules in neuronal development and function *in vivo*** by anatomical and histological analyses and detailed evaluation of the diseased neurons derived from the knockout mouse models.

The laboratory and scientific infrastructure needed for this project is available at the [redacted] Department (Utrecht University), which makes this research highly **feasible**. In addition to its scientific achievements, the group has a long-term internationally recognized track record in basic and translation neuroscience research. This is illustrated by the fact that the group has published numerous papers on this topic (many in high-impact journals), received (inter)national funding (several ERC starting and consolidator grants) and is frequently asked to share data at leading international conferences. The group is part of several international consortia and has international collaborations with other groups.
Based on our previous work and experience with the described research strategy (3.4), our know-how, and the available infrastructure as summarized above, we are confident to investigate the number of molecules stated above to address the listed research objectives within the coming five years. The aimed numbers of candidate molecules to screen, to characterize *in vitro* and *in vivo* is based on our extensive experience in the last years with comparable research strategies and therefore also feasible **within the time-frame** of this project.

The **relevance** of our expected results is explained in 3.3.

**In 3.4 (research strategy) we give a definition of the terms "*in vitro*", "*ex vivo*" and "*in vivo*" used within this proposal and the animal procedure appendix 1.

3.3 Relevance

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

Our results will be a **relevant** contribution to the better understanding of transport and cytoskeleton regulation mechanisms, as well as synapse organization, since the identification and characterization of novel cytoskeleton interactors, motors and regulating molecules will help us to understand how these interactors and molecular changes affect neuronal development and intracellular functions in health and as well as in disease/ LOF situation. The evaluation of LOF/ diseased neuron phenotypes and the identification of pathogenic mechanisms implied in neuronal malformations/ malfunctions in mouse model systems will help to provide the missing knowledge of the molecular and cellular mechanism underlying these processes and help to develop new therapeutic strategies to reverse these phenotypes in the future.

In the research field of neurodevelopmental and neurodegenerative diseases, there is a general agreement that the currently available pharmacological strategies are insufficient. Therefore, these interventions will not lead to a level of repair that is required to allow patients to return to a normal life. Hence, the long-term aim of the research in this field should be the development of effective therapeutic strategies. However, therefore it is essential to better characterize and understand the affected molecular players and molecular pathways and/ or to newly identify molecular cues that can aid in restoring neuronal health. As described above (section 3.1), defects in microtubule-associated and regulating proteins, motor proteins or proteins regulating motor activity and cargo binding are an important and abundant cause of neurological disorders. Knowledge about these molecules and their function, at molecular and physiological level, will provide a starting point for correcting the pathological effects of these molecular players in disease. Our research is aimed at providing this knowledge.
For example, in the past we have determined [redacted] as well as microtubule depolymerizing activity of a subset of kinesin motors ([redacted]). The potential of such fundamental scientific knowledge to be translated towards treatment of disease is supported by our own findings and the research of other groups. For example, mutations in the human [redacted] gene are thought to be associated with neuropathological defects observed in [redacted] patients. Following the research strategy described in detail below (section 3.4), we are confident that based on our knowledge gained from the *in vitro* studies, we can also dissect the physiological role of [redacted] *in vivo* and the cellular mechanisms which are regulated by this molecule in health and disease. This study exemplifies how fundamental knowledge of motor protein regulating molecules can facilitate the design of experimental strategies for *in vivo* analyses, aimed to understand and to treat CNS developmental and degenerative diseases.
In conclusion, this project is important because it will result in fundamental insights into the mechanisms that control cytoskeleton function and synapse transport that if perturbed induce developmental and degenerative neurological disease. These fundamental insights will provide potential therapeutic targets for more successful therapeutic intervention in the future.

3.4 Research strategy

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

To achieve our aims we make use of a seven-step strategy that includes (1) identification of molecules which are differentially expressed in developing neurons using mass spectrometry analysis of rat hippocampal neuron cultures at different time-points of neuronal development and might be involved in the regulation of cytoskeleton dynamics and transport mechanisms, (2) bioinformatics analysis of acquired protein datasets in combination with published data to identify the most promising candidates, (3) testing the impact of candidates on cytoskeleton dynamics and/ or transport mechanisms in rat hippocampal and cortical neurons by suppression via RNA interference (RNAi) using vector based shRNA screens *in vitro***, (4) functional tissue culture and biochemical assays to characterize the role of candidate molecules *in vitro*, and (5) analysis of the effect of manipulation of candidate molecules in nanoscale resolution over time (super-resolution live cell imaging) *in vitro*, or (6) analysis of the effect of manipulation of candidate molecules in genetically modified mouse models *in vivo*** by histological and anatomical analyses and (7) *ex vivo*** in organotypical tissue cultures. Step (7) includes the establishment of a new *ex vivo* approach in our lab and the revealing the fundamental mechanisms of cytoskeleton reorganization in neuronal polarization and migration in detail and over time (live imaging) in organotypical brain slices (*ex vivo***) to fill this gap of knowledge in the field and as basis for our investigation of candidates. Our approach has shown to result in valuable fundamental knowledge and to form a foundation for improved understanding of cytoskeleton remodeling and regulation of transport mechanisms which are of key importance for neuronal development in health and disease.

For all steps, decisions will be made regarding which molecule, molecular and cellular mechanism, cellular compartment (e.g. axon or dendrite), cells or brain region and time-point of neuronal development to focus on. These decisions will be based on data acquired in previous steps or on data acquired in previous research of our lab or our collaborators, e.g. on expression patterns or function *in vitro*, identified protein-protein interactions and/or regulations, a close examination of available literature

and databases and on the outcome of the planned experiments at the relevant yes-no decision points. The amount of available literature and preliminary data and/or previous research in our lab will differ for each candidate. Importantly to notice, for most candidates, we will start at step 2 and will perform RNAi screens to identify the most promising candidates, followed by the described strategy but will only be able to go until step 4/ 5 within the timeframe of this proposal. However, for 6 molecules which we or our collaborators discovered in the past and where mutation in the gene are (suggested to be) associated with malformations/ malfunctions in the CNS and PNA and/or neurological diseases in humans, we have already generated (conditional) knockout mouse lines and are already at step (6)/(7) and will analyze the functional role *ex vivo* and *in vivo* and by anatomical and histological analyses (see also figure 2 for an indication how many candidates will be analyzed at the different steps and go through multiple steps within the time-frame of this project).

Thus, the overall strategy of this project consists of a number of distinct steps aimed at the identification of molecules involved in cytoskeleton dynamics/ reorganization and transport mechanisms in neuronal development using molecular screens and bioinformatics, candidate molecule identification and testing using RNAi screens, functional *in vitro* (and biochemical) assays and super-resolution analysis over time, investigation of the *in vivo* role at the anatomical and cell-functional level, and investigation of the general cellular mechanisms of neuronal development and migration and the functional role of selected candidates *ex vivo*. However, this strategy is a continuation of the work currently ongoing in the lab and **within the coming five years**, we will not go through all steps for any of the investigated molecules.

Figure 1 is a graphical abstract of the logical structure of the different project components/ steps, depending on the feature of the molecule. Go (indicated in green)/no-go and direction decision for the next step will be mainly made based on: a) the expected function in neurons because of published function in non-neuronal cells, previous data and/or protein or protein-domain homology and expression profiles b) the severity of the phenotype in the screens and in the following functional assays *in vitro* (as indicator for the importance), c) the novelty of the molecular function in neurons, d) the specificity of the function, e) its unknown physiological role in health and disease in the nervous system, f) a possible link to neuro-developmental or -degenerative diseases.
The numbers of candidates we aim to analyze in the next 5 years are indicated in italic black. The (animal) model system of each step is underlined.

Figure 2 indicates how many candidates will be analyzed at the different steps of the project and go through multiple steps within the time-frame of this project, or went through certain steps already in the past/ are expected to pass to the next step only after the time-period of this project.

In 3.4.2 we describe in detail how we execute each of these steps and what is required in terms of types of animal experiments.

**We define and use the terms "*in vitro*", "*ex vivo*" and "*in vivo*" within these documents in accordance with the scientific language and practice in the field as following:

- "*in vitro*": dissociated neurons in culture. Gene modifications are in most cases introduced by knockdown (=reduction, no null-allele) or overexpression with help of DNA transfection. However, within this project we also culture and analyze dissociated neurons derived from genetically modified (ko) mice.
- "*ex vivo*": organotypical brain slice tissue cultures. Here the neurons are still in the context of the tissue, but cultured in the incubator. Gene modifications are in most cases introduced by knockdown (=reduction, no null-allele) or overexpression with help of DNA transfection (step7a in fig1). However, we will also use brain tissue from ko mice (step7b).
- "*in vivo*": in contrast to vector-based gene suppression in dissociated neurons, the genes are already deleted/changed in the living organism, in our case in the genetically modified mouse models (=knockout or knock-in mice). This allows to study the function of a certain gene within the entire context of all interacting, counteracting or compensating molecules and factors in the developing organism and therefore to investigate the physiological role of the gene. Even if the analyses of the mice occur after killing of the animals by histological and anatomical analyses - which is the only "*in vivo*" approach in this proposal – this is still referred in the field as an analysis of the "*in vivo*" situation.

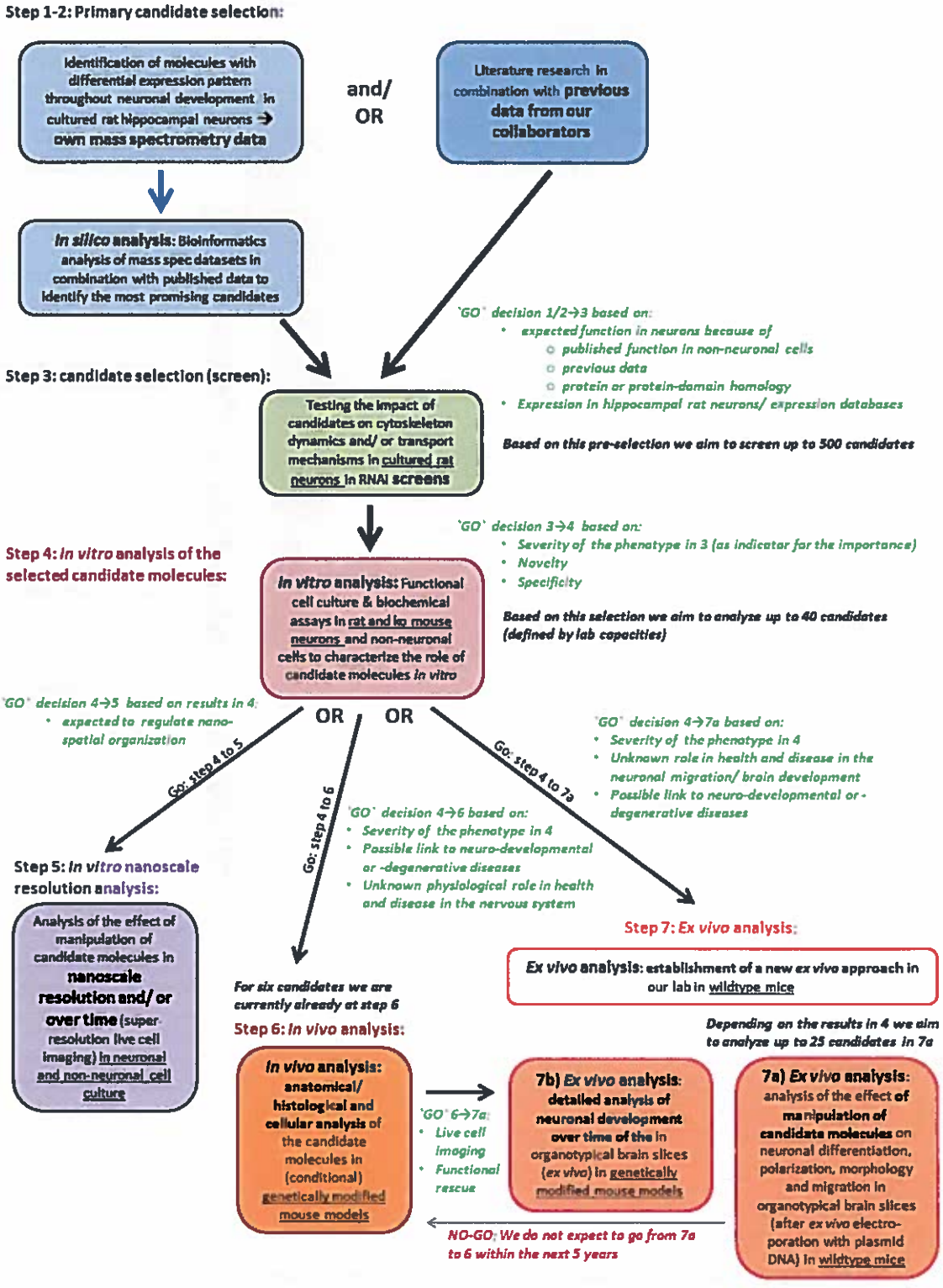

Figure 1. Graphical abstract of our research strategy.

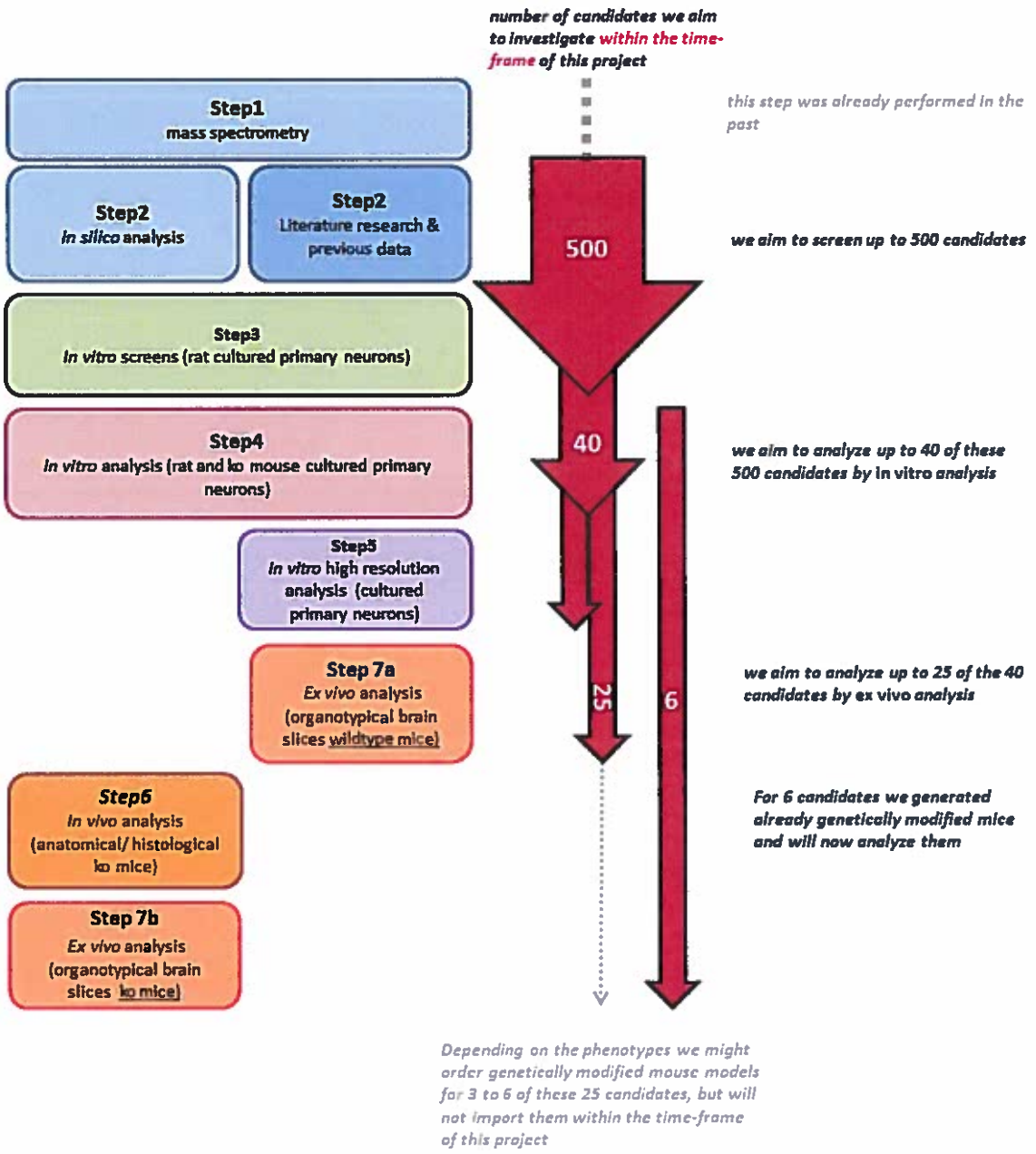

Figure 2. Number of candidate molecules we will investigate in multiple steps of our project within the coming five years.

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.

Description of the different components and the animal experiments linked to these steps (steps are indicated):

Step 1 & 2: In silico *analysis:* The mass spectrometry analysis of primary rat hippocampal neuron cultures at different time-points of neuronal development to identify molecules which are differentially

expressed in developing neurons using and might be involved in the regulation of cytoskeleton dynamics and transport mechanisms are already performed and the datasets are available in our lab. Bioinformatics analysis of acquired gene and protein datasets in combination with published data will be used to identify the most promising candidates. No animals are needed for these steps since the mass spectrometry on the cultured neurons has already been performed (step 1) and the analysis of these data in combination with literature research (step 2) occurs *in silico* (see also fig 2).

Step 3: RNAi screens in hippocampal rat neurons: To test the molecular and cellular role and importance of the candidates selected in step 1 & 2 we will perform multiple knockdown screens, using a vector based shRNA approach. Depending on the scientific question and which aspect of cytoskeleton dynamics/ remodeling or transport will be addressed, both, the candidates to test and the experimental read-out will differ. However, all experiments will be performed *in vitro*, meaning in cultured primary rat hippocampal or cortical neurons, which will be transfected at different time-points to suppress the genes of interest. Most of the screens will be medium size screens, where we test between 20 and 100 candidates, depending on the specific mechanism we want to address. Strikingly, even if we pre-select these many candidates for a specific research question in step 1 & 2, our experience of the last years has shown that only 1 to 4 are indeed of key importance as well as high specificity for the distinct cellular process which was addressed with the screen. As such, our RNAi screens with relatively high number of pre-selected candidates proved to be a powerful tool to find molecules of key importance, and are at the same time still of scientific novelty. Due to the characteristic morphology of neurons and the remarkable importance of intracellular transport and dynamics of the cytoskeleton in the establishment and maintenance of this morphology during development, molecules involved in the regulation of these cellular processes are of unique importance in neurons. Remarkably, many candidates we are analyzing are exclusively expressed in neurons, or are expressed in multiple cell types but have a differential and unique function in neurons. Therefore, neuronal tissue from animals is needed to generate primary neuron cultures, which can be transfected in culture with the shRNA vectors to suppress the genes of interest. Since neurons are post-mitotic cells we depend on primary neuron cultures and will use hippocampal and cortical tissue isolated after killing from rat embryos at E18-E19 ("*in vitro*" approaches in appendix/ procedure 1***). These cells can be transfected at different time points during development *in vitro* and analyzed with question-specific methods, such as specific immunocytochemistry analysis or live cell imaging of fluorescent-labeled cellular markers, observed under advanced microscopes. This analysis provides insight into the key molecular players that control cytoskeleton dynamics and transport and with our previous experience with selecting candidates from molecular screens we aim to identify 30-40 out of initially 500 candidates for detailed functional analysis in the coming 5 years (step 4). This number is - based on our extensive experience with molecular screens in cultured neurons - feasible within the timeframe of this proposal and the actual/ expected size of our research group.

***Please note that we describe in appendix 1 the only animal procedure - which is `killing` for the purpose of tissue isolation for all steps and experiments of our research strategy - the three different approaches "*in vitro*", "*ex vivo*" and "*in vivo*" as description of the experiments which **follow the killing** within different sections, headed with "*in vitro*", "*ex vivo*" and "*in vivo*". Importantly, the investigation of **one** molecule and the investigation of one of our research questions involves **multiple of these approaches**.

Step 4 & 5: (4) functional tissue culture and biochemical assays to characterize the role of candidate molecules in vitro *and (5) super-resolution analysis for selected candidates:* Following the identification of most interesting candidate molecules from the RNAi screens, we will use different techniques (e.g. immunohistochemistry, live cell imaging, [redacted], super-resolution microscopy, Western blotting) to investigate the precise function of these molecules in neuronal development. This step will be also performed in primary hippocampal and cortical neurons, isolated after killing from rat embryos at E18-E19 ("*in vitro*" approaches in appendix/ procedure 1) transfected at different time points during development *in vitro*. These functional analyses will be the main part of our project, and 30-40 candidates is, based on our experience, a feasible number of molecules to analyze in detail in a period of 5 years. To discover the molecular function, we will perform RNAi based gene down-regulation or CRISR/Cas9 based gene depletion; rescue experiments to prove the specificity and possible pathways; biochemical interaction studies; special transport assays (live cell imaging) with fluorescent-labeled markers; treatment of the cultured neurons with drugs that regulate cytoskeleton dynamics; or co-

expression of other (modified) gene expression vectors. The phenotypes will be analyzed with the methods stated above, dependent on the specific experiment and research question. These *in vitro* studies in dissociated neuron cultures will allow us to discover the molecular and cellular function of the candidates in a very time and resource efficient manner, since this model system allows comparable modifications of gene expression and function by transfection with suitable DNA vectors and/or treatment with drugs. Moreover, dissociated neurons in culture are suitable for super-resolution microscopy and we will be able to reveal molecular localization and function on a nanoscale level. Due to our experience and expertise in the analysis of transport and cytoskeleton dynamics in neurons, we have different established neuron culture and biochemical assays to determine whether, and if so how, molecules affect cytoskeleton based transport, cytoskeleton remodeling, neuronal morphology and polarization and additional related parameters. However, we constantly work on the improvement and the establishment of new assays and especially new imaging methods with advanced microscopes to receive new insights which were not possible with the technics used before. For all these experiments, we need to culture primary neuronal cells derived from rat embryos at E18-E19 ("*in vitro*" approaches in appendix/ procedure 1).

Step 6: analysis of the effect of manipulation of candidate molecules in (genetically modified) mouse models in vivo*:*
For some of the most promising candidates, which also shown/ suggested to be associated with malformations/malfunctions in the CNS and PNA and/or neurological diseases in humans, we will use genetically modified mouse models to analyze the role of these candidates *in vivo*. The depletion of selected genes in the living animal (= ko mouse) mimics the genetic situation found in humans where mutations in these genes lead to a complete LOF of the molecule. This results in malformations/ malfunctions in the nervous system and/or neurological disease and thereby generates 'diseased' neurons and brains for our research. In contrast to knockdown experiments in dissociated neuron cultures, this will allow us to unravel not only the molecular, but also the physiological role of the selected gene in in intact nervous tissue in health (wildtype situation) and disease (knockout situation). This fundamental understanding of the physiological role**** of the genes is essential for any therapeutically approaches, and with our research we aim to contribute to the understanding of several malformations/ malfunctions and/ or (neurological) diseases associated with mutations in the specific genes in humans. To accomplish this, we will perform anatomical and histological analysis of the genetically modified mice to analyze neuronal development in physiological conditions ("*in vivo*" approach in appendix/ procedure 1). In specific cases, neuronal cells from the genetically modified mice will be isolated and cultured (appendix/ "procedure" 1). For both anatomical and histological analysis, tissue will be obtained from mice at different developmental stages. To minimize discomfort for the animals, we will perform drop fixation of the tissue isolated after killing the animals for all histological experiments in place of perfusing the (pregnant) animals. The effect of manipulating the genetical code and as such the expression of a certain molecule will be analyzed at the level of neuronal differentiation, neural cell morphology and polarization, and neuronal migration. To minimize the number of laboratory animals used for experiments, we will also fix and freeze non-neuronal tissue of the animals for eventual later anatomical analyses. Using our decision strategy (3.4.1), we aim to analyze up to 6 candidate molecules by detailed *in vivo* analysis. Again, this number is based on available infrastructure and previous experience. Type of animal experiment: killing of mice, followed by tissue isolation, fixation and histological and anatomical analyses ("*in vivo*" approach in appendix/ procedure 1) or neuronal cell cultures derived from the genetically modified (ko) mice ("*in vitro*" approaches in appendix/ procedure 1).
The (conditional) genetically modified mice for these 6 candidates are already generated and established lines. Our previous experience with these mouse lines showed that the genetic modification does not cause discomfort for the animals in heterozygous status and in homozygous status before birth in any of the mouse lines. We will be able to maintain all the mouse lines and perform all experiments with animals without (or before on-set of) discomfort for the animals (see ("*in vivo*" approach in appendix/ procedure 1 for details).
Within the timeframe of this proposal, we do not expect to reach step 6 for any of the newly identified molecules and characterized in step 2 to 4/ 5 since from our experience the identification and detailed *in vitro* analysis alone already takes 2-4 years and the generation of a new knockout mouse model takes at least 2 years. Thus, for the most interesting candidates that we will select in step 2 to 4 in the coming

five years, we may still make the decision to go further to step 6 and to generate a knockout or knockin mouse model; however, we won't be able also to implement that within time-frame of this proposal.

Step 7: analysis of the effect of manipulation of candidate molecules in the developing brain ex vivo: For several of the selected candidates in step 3 by using our decision strategy (3.4.1), we will also analyze neuronal development and migration in the neocortex over time by performing *ex vivo* brain electroporation with vectors to manipulate/ drive gene and/or marker expression, followed by culturing organotypical brain slices (short name: "*ex vivo* approach"). To do so, we first will establish this *ex vivo* approach in our lab and aim to reveal the fundamental mechanisms, especially of cytoskeleton regulation in neuronal polarization and migration in the cortex in detail and over time. This first step is essential to understand these mechanisms in detail and especially in a continuous time frame (live imaging) to fill this gap of knowledge in the field and to obtain the knowledge of the normal (wildtype, or unmodified) cellular behavior in the brain as basis for our investigation of the selected candidates.
Using our decision strategy (3.4.1), we aim to analyze up to 25 candidates (including several of the candidates chosen also for *in vivo* analysis) in 5 years. Since this is a new approach in our lab which we are just setting up now, this number is based on previous experience of the postdoctoral researchers in our group, gained at other laboratories and on the pilot experiments performed so far. Type of animal experiment: killing of mice, followed by *ex vivo* brain electroporation with vectors in embryos (E14), culturing of organotypical brain slices and (live cell imaging) analysis of the neuronal cells in the brain tissue ("*ex vivo*" approach in appendix/ procedure 1).

****We follow in our documents the definition of physiology (from Ancient Greek φύσις (physis), meaning 'nature, origin', and -λογία (-logia)), meaning that 'study of' is the scientific study of normal mechanisms, and their interactions, which works within an intact living organism. Its focus is in how organisms, organ systems, organs, cells, and biomolecules carry out the chemical or physical functions that exist in an organism. Thereby, these functions can also by analyzed *ex vivo*/ post mortem, e.g. by histological and anatomical analyses (to study e.g. the result of cellular migration during development).

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.

In this project, we characterize molecules that regulate cytoskeleton dynamics and organization, cytoskeleton based transport and synaptic organization using bioassays and *in vivo* and/ or *ex vivo* models.
Typically, the strategy we use consists of five to seven steps that logically follow each other and progress from 'candidate molecule identification' to validation in an animal model (*ex vivo* and/ or *in vivo*, see also figure 1 above). For each candidate, a decision is made whether, or not the candidate should be studied at the next step. Primary candidate selection is in part based on the differential expression pattern of the molecules in cultured rat hippocampal neuron cultures found by mass spectrometry analysis in our lab, by our collaborators or other. Depending on the molecules, all seven steps described above can be followed, for most of the molecules however we will follow only a subset of five to six steps. This selection will be based on: a) the expected function in neurons because of published function in non-neuronal cells, previous data and/or protein or protein-domain homology and expression profiles b) the severity of the phenotype in the screens and in the following functional assays *in vitro* (as indicator for the importance), c) the novelty of the molecule function in neurons, d) the specificity of the function, e) its unknown physiological role in health and disease in the nervous system, f) a possible link to neuro-developmental or -degenerative diseases (see figure 1).
Our strategy is very ambitious and the different components can take for one molecule also more than five years. The strategy described above has already allowed us to identify a number of candidate molecules, which are currently now already in step 6 (e.g. [redacted]). In the coming five years, we expect to identify more molecules which we aim to investigate on all levels: *in silico*, *in vitro*, *ex vivo* and *in vivo*. However, due to the capacities of our lab and the time which is required to generate a knockout mouse (around 2 years between the order and the possible import), we do not expect one of the candidate molecules that we will newly identify and characterize to also reach from step 4 or 7a to step 6 within the time-frame of this proposal (see also fig. 2). Therefore, we do not expect to import new (knockout) mouse lines for additional molecules in the coming five years which might have discomfort. (We might however import mice without discomfort for the breeding with the already established mouse lines which

result in offspring without discomfort and won´t be an animal procedure therefore.) We are however confident that we can successfully establish the *ex vivo* approach as a reliable method in our lab and that we will be able to use this technique for analyzing the role of the 25 molecules in an organotypical situation which we selected on the selection point from step 4 to 7a. This implies that for newly identified molecules we will perform step 7a before step 6 and will only go on to step 6 in the future if we expect to gain substantial and required additional information from the *in vivo* (knockout) model in comparison to the *ex vivo* model.

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	Elucidation of fundamental cell and molecular mechanisms underlying cytoskeleton dynamics, transport, synapse organization and neuronal specification and migration in rodent neurons *in vitro,* in organotypical brain slice cultures *ex vivo* and *in vivo* by histological/ anatomical analysis of genetically modified mice models.
2	
3	
4	
5	
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10	

Centrale Commissie Dierproeven

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, 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'.	10800	
1.2	Provide the name of the licenced establishment.	University Utrecht	
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number	Type of animal procedure
		1	Elucidation of fundamental cell and molecular mechanisms underlying cytoskeleton dynamics, transport, synapse organization and neuronal specification and migration in rodent neurons *in vitro*, in organotypical brain slice cultures *ex vivo* and *in vivo* by histological/ anatomical analysis of genetically modified mice models.

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 procedures described in this project are based on a large body of scientific literature in the field and on our own experimental experience. These animal studies are performed worldwide and therefore make a translation and extrapolation of data between research groups feasible.
Our project examines the fundamental cell and molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton transport and synapse organization during neuronal development. The main goal of our project is first to identify molecules of key importance, or so far unknown or poorly understood function in neurons (e.g. microtubule motor protein regulating molecules); and then to investigate and understand their mechanisms-of-action that regulate these crucial cellular processes.
In order to identify molecules involved and to understand the function of these molecules, molecular screens and cell molecular bioassays are performed *in vitro*, using primary neuron cultures obtained from wildtype rats or genetically modified mice at pre-natal stages. Since neurons are post-mitotic cells these neuron cultures cannot be maintained as continuous cultures but must be prepared from animal tissue each time. We will refer to these dissociated neuron cultures through the entire document as "*in*

vitro approaches"*.

*Please note that we describe in this appendix 1 the only animal procedure, which is `killing` for the purpose of tissue isolation for all steps and experiments of our research strategy. The three different approaches "*in vitro*", "*ex vivo*" and "*in vivo*" as description of the experiments which follow the killing within different sections, headed with "*in vitro*", "*ex vivo*" and "*in vivo*" approach. Importantly, the investigation of one molecule and the investigation of one of our research questions can implicate multiple of these approaches and they are coherent within the investigation of one molecule.

We define and use the terms "*in vitro*", "*ex vivo*" and "*in vivo*" within these documents in accordance with the scientific language and practice in the field as follows:

- "*in vitro*": dissociated neurons in culture. Gene modifications are in most cases introduced by knockdown (=reduction, no null-allele) or overexpression with help of DNA transfection. However, within this project we also culture and analyze dissociated neurons derived from genetically modified (ko) mice.
- "*ex vivo*": organotypical brain slice tissue cultures. Here the neurons are still in the context of the tissue, but cultured in the incubator. Gene modifications are in most cases introduced by knockdown (=reduction, no null-allele) or overexpression with help of DNA transfection (step7a in fig1). However, we will also use brain tissue from ko mice (step7b).
- "*in vivo*": in contrast to vector-based gene suppression in dissociated neurons, the genes are already deleted/changed in the living organism, thus in the genetically modified mouse models (=knockout or knock-in mice). This allows us to study the function of a certain gene within the entire context of all interacting, counteracting or compensating molecules and factors in the developing organism and therefore to investigate the physiological role of the gene. Even if the analyses of the mice occur after killing of the animals by histological and anatomical analyses - which is the only "*in vivo*" approach in this proposal – this is still referred in the field as an analysis of the "*in vivo*" situation.

For the *in vitro* approaches:

brain tissue will be isolated from wildtype rats at pre-natal stages E18-E19 or from genetically modified mice at E17-E18 and hippocampal and cortical neurons will be cultured for further experiments or used for biochemical analysis.

The tissue obtained from wild-type rats and genetically modified mice are used for:

1. Bioassays using primary hippocampal or cortical neurons in culture to test e.g. axon outgrowth, dendritic development and synaptic morpho-dynamics as read-out parameters for changes in cytoskeleton dynamics, intracellular transport mechanisms and synaptic organization.
2. Manipulation of the cultured cells; for instance through plasmid-driven gene expression or suppression or pharmacological agents and subsequent analysis using the techniques mentioned below.
3. Immunohistochemistry and subsequent imaging with advanced microscopes.
4. Live cell imaging, including motor protein assays.
5. Biochemical analysis (RNA/DNA/protein extraction, mass spectrometry analysis).

With our *in vitro* studies, we aim to identify and characterize 30 to 40 candidates (out of up to 500, see also figure 1 in the proposal) of unique importance for the processes stated above. For 25 of these candidate molecules, we aim to analyze also their role in neuronal migration and neuronal polarization in the context of brain tissue. Organotypical brain slice cultures, described here as "*ex vivo*" approaches where *ex vivo* modifications in gene expression are introduced via electroporation of plasmid DNA, followed by organotypical slice cultures are a valuable tool to elucidate the cellular function of a molecule in certain aspects of development, like neuronal migration or polarization in a "close to *in vivo*" organotypical situation. These *ex vivo* experiments are widely performed, which makes the translation and extrapolation of data between research groups feasible. They are a widely used model system to mimic the *in vivo* development of the brain *ex vivo*.

For the *ex vivo* approaches,

we will electroporate plasmid DNA to drive gene suppression or expression in combination with plasmid DNA to express cellular fluorescent markers into the cells of the ventricular zone of the cortex in isolated heads of E13.5 to E16.5 embryos (=*ex vivo*), section the brain tissue and culture the slices for several

days. Neuronal polarization and migration will be analyzed be immunohistochemistry and by live cell imaging of the fluorescently labeled cells with advanced live cell imaging microscopes. Moreover, we will analyze parameters like cell morphology, transport and microtubule dynamics in single cells in the context of the surrounding cells and extracellular factors in the tissue that build the functional brain with all its neuronal networks and specialized architecture. In most cases we will use wildtype mice and introduce gene expression or suppression by electroporation with plasmid DNA encoding for selected fluorescently tagged molecules or shRNAs (or CRISPR/Cas9) cassettes respectively. We will also use genetically modified mice (without discomfort) for this procedure to analyze the described parameters live in single cells in the brain by live cell imaging of single fluorescently labeled cells.

The tissues obtained from wild-type and genetically modified mice are used for:

1. Immunohistochemistry
2. Fluorescent live cell imaging

With these *ex vivo* approaches specific aspects of neuronal development and neuronal cell fate can be analysed in the context of the intact tissue and even be observed live under the microscope. However, to elucidate the physiological** role of a specific molecule in health and in altered (mutated)/ diseased situations, this model system is no longer sufficient. Here we require an intact organism as model system, thus an *in vivo* model which allows to study the function of a certain gene within the entire context of all interacting, counteracting or compensating molecules and factors in the developing organism and therefore to investigate the physiological role of the gene. Even if the analyses of the mice occur after killing of the animals by histological and anatomical analyses, which is the only "*in vivo*" approach in this proposal, this is still referred in the field as an analysis of the "*in vivo*" situation. The use of genetically modified mouse models to study the physiological function** of a molecule in health and disease *in vivo* is a widely used and accepted model system, also for neuronal diseases in human. The animal studies described in this appendix are widely performed, which makes translation and extrapolation of our data feasible. Here, we use the modification of the genetic code of mice as a valuable tool to identify and characterize the mechanisms underlying neurogenesis, neuronal specification and development, migration and plasticity and related disease.
As such, we will perform anatomical and histological analysis as well as biochemical analysis of tissue isolated from genetically modified mice.

** We follow in our documents the definition of physiology (from Ancient Greek φύσις (physis), meaning 'nature, origin', and -λογία (-logia)), meaning that 'study of' is the scientific study of normal mechanisms, and their interactions, which works within an intact living organism. Its focus is in how organisms, organ systems, organs, cells, and biomolecules carry out the chemical or physical functions that exist in an organism. Thereby, these functions can also by analyzed *ex vivo*/ post mortem, e.g. by histological and anatomical analyses (to study e.g. the result of cellular migration during development).

For the "*in vivo* approaches",
the tissues are harvested from genetically modified mice from pre-natal (E14-E19 depending on the line) or post-natal stages (P0 until 30 weeks), depending on the research question and on the assay performed. Several, but not all of the selected candidate molecules are exclusively expressed in neuronal tissue. To minimize the number of laboratory animals used for experiments, we will also isolate non-neuronal tissues that also express these molecules of the animals for later anatomical analyses.
Neuronal and other tissue will be isolated from mice at the different developmental stages stated above after killing of the animals. To minimize discomfort for the animals, we will perform only drop fixation of the tissue isolated after killing the animals for all histological or anatomical experiments, in place of perfusing the (pregnant) animals. We developed histology protocols which allow us to get results of high quality with drop-fixation of the tissue, allowing us to avoid perfusion of the animals.
The effect of manipulating the genetically code will be analyzed mainly, but not only, at the level of neuronal differentiation, neural cell morphology and polarization, neuronal migration and changes in protein expression.

The tissues obtained from wildtype and genetically modified mice are used for:

1. Immunohistochemistry, anatomical analyses, 3D-imaging of solvent cleared organs, or *in situ* hybridization staining
2. Biochemical analysis (RNA/ DNA/ Protein extraction and probing, mass spec analysis).

Using genetically modified mice as *in vivo* models for the selected candidate molecules includes the maintenance of these mouse lines without discomfort. Over the last few years, we already generated and established or obtained genetically modified mouse lines (knock-outs, conditional knock-outs or knock-ins) for 6 candidate molecules and maintain now these lines including Cre-recombinase expressing mouse lines.
Due to our experience with these mouse lines in the past, we know that the genetic modification does not cause discomfort for the animals for most of the lines. In cases where it causes discomfort for the homozygous (knockout) mice, we will apply advanced breeding procedures to exclude discomfort:

- Conditional knockouts: crosses between mice carrying floxed alleles and Cre-expression mouse lines can lead to the generation of conditional null-mutant mice. As a consequence of this advanced breeding procedure, mice will only have a gene deletion in a particular cell type. We already generated conditional mouse lines and will maintain these mouse lines were the animals showed to have no discomfort.
- Heterozygous breeding regime: the heterozygous mice have no discomfort and are comparable to their wildtype littermates. Therefore, we will keep some mouse lines in a heterozygous state only by breeding heterozygous mice with wildtype mice. However, for experiments we will breed two heterozygous animals and kill the offspring and isolate the tissues for further experiments at embryonic states (E14-E19), where animals are shown to have no discomfort yet.

For all approaches of our project,
animals will be killed according to Annex IV of directive 2010/63/EU and tissue will be isolated after killing for further experiments (see below).

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

A. For the ***in vitro* approaches** which depend on primary neuron cultures or brain tissue lysates isolated from developing wildtype rat or genetically modified mouse embryos, pregnant mother animals will be killed by CO_2/O_2 suffocation. The CO_2 will be used in gradual fill in accordance with the Directive 2010/63/EU, Annex IV. The death of the animals is monitored with the highest accuracy and death will be ensured by additional cervical dislocation. The embryos will be quickly taken out of the uterus, put on ice-water and are subsequently decapitated***. Brains will be isolated and kept in cold buffer on ice, and tissue will be harvested for hippocampal and/or cortical neuron cultures or biochemical analyses.
B. For the ***ex vivo* approach** performed in embryonic brains of wildtype and genetically modified mice, pregnant mothers will be killed by CO_2/O_2 suffocation or cervical dislocation. The CO_2 will be used in gradual fill in accordance with the Directive 2010/63/EU, Annex IV. The death of the animals is monitored with the highest accuracy and death will be ensured by additional cervical dislocation. For harvesting tissue from embryos for the experiments, the embryos will be quickly taken out of the uterus, put on ice-water and are subsequently decapitated***. Brains and other tissue will be isolated from the embryos (and adult animals if required) and kept on in buffer on ice and used for further analyses by the *ex vivo* approach (or histological or biochemical analyses of the tissue obtained from the adult animals).
C. For the ***in vivo* approach** where genetically modified mice will be analyzed by anatomical and histological analyses, adult animals older than P14 will be killed by CO_2/O_2 suffocation or cervical dislocation. The CO_2 will be used in gradual fill in accordance with the Directive 2010/63/EU, Annex IV. The death of the animals is monitored with the highest accuracy and death will be

ensured by additional cervical dislocation. For the anatomical/histological analyses of embryonic tissue, first pregnant mothers will be killed as described. The embryos will be quickly taken out of the uterus, put on ice-water and are subsequently decapitated***. Brains and other tissue will be isolated from the embryos (and adult animals if required) and kept on ice in cold buffer and used for anatomical and histological analyses. For experiments where neonate pups younger than P8 will be used for experiments, the neonates will be killed as described before for the embryos; they are put on ice-water and are subsequently decapitated***.

*** Decapitation is used because other methods are not suited for embryos and very young pups. Cervical dislocation by squeezing the neck behind the occiput of the cranium to dislocate the atlanto-occipital joint is not recommended for embryos and neonate pups because they are still too small and the mentioned structures are not sufficiently developed to make cervical dislocation a fast and efficient method of killing. Instead, it increases the possibility that the embryos and neonates suffer. Also, carbon dioxide does not lead to a fast and efficient death of foetal and neonate rodents since they are resistant to hypoxia. CO_2 asphyxiation is therefore not recommended for mice embryos and young pups as specified in the requirements of these methods in the table with methods of killing of the Annex IV of directive 2010/63/EU (requirement point 3). Concussion/ percussive blow to the head is also not possible because brain tissue is exactly the tissue we analyze in our experiments (other tissue from embryos is only isolated and analyzed in addition to brain tissue, see also B). Finally, injections with an overdose of anesthetics are not efficient in embryos and pups younger than P8 and will cause more discomfort than decapitation. Since all these methods of killing are not suitable for embryos and young pups in our research, the embryos and neonate pups will be killed by decapitation which is listed in the Annex IV of the Directive 2010/63/EU as method of killing for rodents if other options are not possible (requirement point 12 of the table with killing methods).

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

Qualitative analysis: The number of animals is based upon our extensive experience gained over the last years and are constantly refining.
For instance, **for experiments that require primary neuron cultures or biochemical analysis (*in vitro* approaches)**, the number of animals depends on the total number of cells that can be harvested from the desired brain region. Some brain areas, like the cortex are large and provide large numbers of cells, whereas other brain areas, like the hippocampus are much smaller and provide less cell numbers. However, the cells isolated from the hippocampus are much more homogeneous than cortical neurons (about four different types of neurons versus hundreds different types) and reduce the variability and the required amounts of neurons which have to be analysed and the number of experiment replication which is required in order to get statistically significant data. Therefore, we use hippocampal neurons for most experiments listed above. We only use cortical neurons for some biochemical assays or studies of the genetically modified mice. Even if the hippocampus is smaller than the cortex, the number of cells we can isolate from one embryo is in most cases enough for several experiments. We take strict care that all isolated neurons will be divided among the investigators in the lab and that they will all be used (see also below in D, descriptions for "refinement" in the 3R´s). For the experiments where cortical neurons will be used, no additional animals are required since we will isolate in this case hippocampus and cortex from the same embryo. In each case, the experimental design further dictates the number of cells needed.
Quantitative analysis: Our long-term experience with neuron cultures allows us to estimate the required numbers of neurons per experiment very well. For each experiment in standard screens, one pilot experiment will be performed before we perform three experiments under the optimized, constant conditions (N=3). Our experience with molecular screens in neurons in the last years showed that these pilot experiments help to reduce the total amount of neurons needed for one specific approach. Because even though the general techniques (like hippocampal neuron cultures, neuron transfection or immunocytochemistry) are operational in the lab, the read-out parameters for the individual screens are highly dependent on the specific research question and can be unique for the individual screen. And even if the read-out parameters are similar between different screens, the actual settings still depend on the analyzed candidate molecules****, the degree of severity and the variation of the phenotypes. For technically more challenging experiments with multiple variable parameters, or experiments with more

biological variability (e.g. transport in living cells), up to two pilot experiments will be performed to optimize the experimental conditions corresponding to the specific molecule which will be analysed in the assay and to evaluate the extent of variation and perform statistical analysis (a power analysis) to ensure that we use the least amount of animals possible per group that will be required to reach statistical significance.

**** the knockdown of certain molecule can e.g. impair the viability of the neurons in general and therefore demand a shorter duration of the experiment or the use of an inducible promotor.

The number of animals for the ***ex vivo* approaches** is based upon our experience with *ex vivo* experiments performed in our lab so far as well as upon the experience of researchers with this technique elsewhere. In order to reduce the variation between the experiments due to technical issues, we will still perform several pre-experiments / pilot studies to optimize the experimental setup and to make more precise power-calculations for our experiments. These pre-experiments will not have to be performed for each assay, but are rather required to fully establish this new technique in our lab and to generate a reliable control data-set as standard for the following assays with experimental conditions (e.g. gene knockdown or overexpression). Due to the high technical challenge of this technique, the pre-experiments are still required to optimize our protocol and to reduce the variability accruing from technical rather from biological issues. Thus, these pilots will need several animals in the first year, but will help us to reduce and minimize the total number of animals in the coming five years.
For the estimation of the number of animals we will take the following statistical parameters in account:
The variance, due to the normal biological variance observed in *ex vivo* experiments and due to the complicated technical setup with multiple factors which must be controlled is relatively high when compared with *in vitro* studies. Moreover, we expect the phenotypes to be subtler for several outcome parameters for some candidate molecules. However, phenotypes with a 20% change in relation to the controls have also been shown to be significant and physiologically relevant in the past. Based on these factors and on our experience, we expect to need a maximum of N=14 per assay to reach significant relevance with a p-value below 0.05 which we set as minimum for this approach. Due to the thickness of the slices and the size of the brains, one brain can be used for one experiment, thus 14 embryos are needed for one assay with an experiment replication of N=14. We aim to get these N=14 from at least 3 different litters. However, since we induce the modification in gene expression by electroporating plasmid DNA, we can use the embryos of one litter for different assays, even for the investigation of different candidate molecules by introducing the desired DNA. We expect to have on average 7 embryos per litter and will coordinate the experiments in a way that all embryos of all litters will be used for experiments and no `surplus` embryos must be killed.

The number of animals for the *in vivo* approaches is based upon our experience in the work with genetically mice in general and the mouse lines for the candidate molecules stated above in particular. For experiments with genetically modified mice the number of animals depends on the amount of knockouts (knockins) versus control animals we have per litter, as well as on the amount of tissue that can be harvested from the desired region of the central nervous system at a certain stage of development/ age. To make optimal use of the isolated tissues for immunohistological experiments, we normally collect the tissue as non-continuous series of sections on several coverglasses, which allows us to stain the tissue from one animal and organ with different antibody combinations and to address different research questions with the tissue isolated from one animal. However, at early stages of development, some brain areas are still very small and will provide smaller numbers of sections.
Quantitative analysis: Our experience with this procedure allows us to estimate the required numbers of neurons per research question and for some experiments we already performed pilot experiments to calculate the number of animals needed. In all other cases, we will perform one pilot experiment to evaluate the extent of variation and perform the statistical analysis to ensure that we use the minimum number of animals per group required to reach statistical significance and to optimize the experimental conditions corresponding to the specific assay. For the heterozygous breeding regime, we will breed heterozygous mice to avoid discomfort and expect 25% knockout embryos per litter. One litter contains on average 7 embryos. For histological and biochemical assays performed with tissue from genetically

modified mice we count one embryo as N=1 and aim to analyse at least an N=5 from at least 3 different litters for each genotype in accordance with good scientific practice in the field. Our experience in the past showed that an N=5 is required and sufficient to reach statistical significance and reliable results for experiments where tissue from genetically mice will be analysed. Therefore, 4 litters are needed for each assay. For the *in vivo* study of the molecules which would cause discomfort and lethality if globally depleted, we will work with conditional knock-outs to avoid discomfort. Here, we can choose a breeding regime where we expect 50% knockout and 50% wildtype littermates. For lines where the knock-out has no discomfort we can use a breeding regime where we expect 50% ko and 50% wildtype littermates. For the mouse lines for these genes we will need therefore normally minimal 3 litters per experiment for assays with this procedure.

B. The animals

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

Species: We will use wildtype rats (*Rattus norvegicus*) for *in vitro* approaches in step 3 to 5 of our project, wildtype mice (*Mus musculus*) for the *ex vivo* approach in step 7a, and genetically modified mice (*Mus musculus*) for *in vitro* experiments in step 4, the *ex vivo* assays in step 7b and for the *in vivo* approach in step 6.
For our *in vitro* approaches, where primary hippocampal and cortical neuron cultures derived from wildtype animals are used, we use rat brain tissue. Rat neuron cultures are a reliable, widely used and accepted model system for the parameters we will analyze. Rat brain tissue, rather than mouse brain tissue, is the preferred animal tissue here, because one rat embryo at E18 yields nearly two-fold the amount of hippocampal and cortical neurons that can be harvested and cultured in comparison to one mouse embryo at the corresponding stage. The rat brain structures which are harvested for neuron isolation are bigger in size and the rat neurons are more robust compared to mouse neurons and show therefore a higher survival rate.
For the *ex vivo* approaches and the analyses of knockout or knockin animals, we use mice rather than rats, even if the amount of tissue we can harvest is lower. Mice are the most commonly used model system in the field in general and for the assays we perform in particular. The large majority of the rodent models in neuroscience are mice, while rats are (still) very uncommon. This general preference for mice to rats is explained by pragmatic rather than scientific arguments; mouse colonies are cheaper to maintain due to the smaller size of the animals. We decided mice models since only this allows us to directly compare our results with the results of other research groups and other mouse models in the field and to extrapolate our findings. Especially for experiments which address neurodevelopmental research questions it is very important to perform the experiments at exactly the same life stage of the embryos to allow a direct comparison. Due to the slightly shorter pregnancy of mice compared to rats, the data of these two species cannot be compared directly.

Origin: The wildtype rats and wildtype mice are obtained from licensed laboratory animal companies; the genetically modified mice are bred in the GDL.

The estimate of the total number of animals is primarily based on our experience over the past years. For the different steps in our project and the specific experiments at these steps, we will use specific calculations, of which a very **general outline** and factors taken in account is given below. A more detailed explanation and **the corresponding example calculation are given as supplementary information at the end of this document**. In our application, we describe how we select candidate molecules, how we will analyse their role during neuronal development and how many molecules we aim to study. This number and the specific assays we plan to use determine the total number of animals used.
Time-bred adult animals give us the opportunity to harvest embryos at the desired stages. In most cases, the mothers are young animals of approximately 10 to 20 weeks old. In quick succession, the pregnant mothers will be killed as described above, embryos will be removed from the uterus and killed, and the embryonic brain tissue isolated for the hippocampal and/ or cortical neuron cultures. Thus, the

number of animals used is the sum of pregnant mothers and embryos.

Estimated numbers and life state for the *in vitro* approaches:
For our *in vitro* studies where wildtype rat primary hippocampal and cortical neuron cultures are used, tissue will be obtained in accordance with well-established and worldwide used protocols from E18-E19 embryos; for studies where neurons derived from genetically modified mouse models are used, tissue will be obtained from E17-E18 embryos. In all cases, we will use both female and male embryos because there are no sex-dependent effects known for the read-out parameters we will measure in our experiments.
General considerations for the calculations for step 3 (*in vitro* screens) performed in rat primary hippocampal neurons: For each experiment in standard screens, one pilot experiment will be performed before we perform three experiments under the optimized, constant conditions (N=3). Our experience in the past has shown that an N=3 is required and sufficient to reach statistical significance and reliable results for most experiments where fixed neurons will be analyzed, if the experimental conditions have been optimized for the specific approach with help of the pilot experiment. If the experimental conditions are optimized for the specific screen, there will be a sufficient number of transfected neurons per experiment (N), e.g. 100 neurons per N that can be analysed in order to reach significant and reliable results.
We aim to screen up to 500 candidate molecules within the time-frame of this project. 400,000 neurons are needed per molecule for an N=3 plus pilot; 200,000,000 for all candidate molecules together (see suppl. information for the corresponding calculations). 286 embryos are required to isolate this amount of primary hippocampal neurons. A normal rat litter contains approximately 10 embryos. Therefore: 286 embryos + 29 adult pregnant females = 315 animals are needed for step 3.
General considerations for the calculations for step 4 & 5 for the *in vitro* analysis performed in rat primary hippocampal neurons: We aim to select up to 40 candidate molecules from the screens. For these, we will first validate the observed results from the screens by rescue experiments in accordance with good scientific practice in the field. 3,200,000 neurons are needed per molecule for the rescue experiments; 128,000,000 neurons for all 40 molecules together. For the validation by rescue experiments we will need, in total 183 embryos + 18 pregnant mothers, thus around 200 rats.
For the actual functional characterization of the validated molecules (step 4) and nanoscale analysis (step 5) at least 2 pre-experiments (pilots) are required to optimize the experimental condition for the specific molecule and to calculate the required number of neurons which must be analysed in order to come to statistically significant results (power calculations) due to the higher technical challenge, multiple potential read-out parameters and the higher variability of the results in living cells. 84,000,000 hippocampal neurons will be used per candidate for the detailed investigation of the cellular and molecular role of these candidates *in vitro*; 3,360,000,000 for all 40 molecules. In total, 4800 embryos + 480 adult pregnant females = concerning these example calculations 5280 rats will be needed for step 4 and 5 of our project. (See suppl. information for the corresponding calculations).
Following these calculations, for all *in vitro* experiments in rat cultured neurons which we aim to perform in step 3, step 4 and step 5 of our project in five years, we need to kill 5269 embryos and the corresponding 527 adult pregnant females, thus, 5796 rats in total to isolate after killing the desired primary hippocampal neurons. However, due to normal technical issues in primary neuron cultures such as contaminations, medium batch dependent variation in the viability and comparable unpredictable factors, our experience from the last years has shown us that we have to calculate an additional 10 % neurons required. Moreover, scientific requirements may change the number of assay we have to perform per candidate molecule slightly. Therefore, we estimate that we will use a maximum of 6500 rats altogether.

Calculations for experiments with the *in vitro* experiments using neuron cultures dived from genetically modified mice: One mouse litter normally contains 7 embryos, for both wildtype and our genetically modified mouse lines. For heterozygous breeds we expect 25% knockout embryos per litter. Our experience in the past has shown that an N=5 (N=1 embryo per genotype) from at least 3 litters is required and sufficient to reach statistical significance and reliable results for experiments where neurons

from genetically mice will be analyzed. We need here an N=5 instead of N=3 due to the lower amount of neurons we can harvest and analyze per embryo compared to rat embryos. Moreover, dependent on the read-out parameter and the severity of the analysed phenotype, the variability in the phenotype within one genotype can be high in some assays, or the differences between the genotypes (e.g. between wildtype and heterozygous or heterozygous and knockout) for the measured parameters can be subtler and therefore require an N=5 in total. Therefore, 4 litters are needed for each assay.
For breeding regimes which yield 50% knockout and 50% wildtype littermates, we will need 3 litters per experiment.
For our *in vitro* studies in step 4, a maximum of 10 assays will be performed for all mouse lines. Calculating for 4 assays with 4 litters and 6 assays with 3 litters each, we will need to kill 4*4+6*3=34 adult, pregnant mothers and estimated 34*7=238 embryos. Thus, we estimate to use in absolute terms 272, but about 270 genetically modified mice for the *in vitro* cell culture experiments at step 3.

Estimated numbers and life state for the *ex vivo* approaches:
We will use embryonic tissue from mice between E13.5 and E16.5 for these experiments and will use again both, female and male embryos (see above). Embryos younger than E14 are not taken into account in our estimations shown below.
Calculations for the wildtype mice used for *ex vivo* experiments in step 7a of our project: We aim to investigate the role of 25 candidate molecules in neuronal development and migration *ex vivo*. With the statistical methods mentioned above, we expect to need here an N=14 (1 embryos = 1 N). Normally, one assay has 2 conditions: modification and control. We aim to perform a maximum of 3 assays per candidate molecule with embryos older than E14. Thus, (25*3)*(14*2)=2100 embryos. With an average of 7 embryos per litter we need to use 300 pregnant adult mice.
Moreover, we aim to perform per candidate molecule a maximum of 2 assays with embryonic tissue from animals younger than E14. Therefore, we estimate to use 200 pregnant adult mice.
For the pilot experiments and in order to reliably establish this method in our lab (see explanation above) we estimate to need a maximum of 30 litters, thus 30 pregnant females and 210 embryos and therefore a maximum of 240 animals.
Taken together, we expect to need a maximum of **2840 wildtype mice** (about 2310 embryos and a maximum of 530 adult pregnant females) in total.

Calculations for genetically modified mice used for *ex vivo* experiments in step 7b: Also here, we expect to need an N=14 (embryos per genotype) per assay. One assay has one condition; functional rescue assays have 2 to 3 conditions. We calculate with the average of 2 conditions per genotype, and aim to perform 2 assays for 3 of the mouse lines. Thus, with 2 genotypes (wildtype and ko) and 2 conditions these are (2*3*3)*14=252 embryos. With an average of 7 embryos per litter and taken into account the unpredictable variation of the genotypes per litter, we estimate to kill a maximum of 50 adult females and therefore around 300 genetically modified mice older than E14 for *ex vivo* assays.

Estimated numbers and life state for the *in vivo* approaches:
We will use embryonic and adult mice. Embryonic mice will be used between E13 and E19; postnatal and adult***** mice between P0 and 30 weeks of life. For the experiments where tissue from embryos will be isolated, we will use female and male embryos, because there are no sex-dependent effects known for the read-out parameters we will measure in our experiments. For the experiments where tissue from adult mice will be used for experiments, we will use both, female and male animals, however, will analyze and compare the data for both sexes separately.
General considerations for the calculations for the *in vivo* experiments (histological and anatomical) using genetically modified mice in step 6: For the *in vivo* analyses, we mainly compare wildtype with homozygous knock-out/ knock-in, which means 2 genotypes per assay. The cut tissue from one animal can however be collected on 4-6 coverglasses and therefore used for different assays. Some glasses with tissue will be needed to test new antibodies for molecule-specific research questions. We will also need an N=5 (see explanations above) from at least three litters for these experiments with tissue from

genetically modified mice and expect 1 litter to have on average 7 embryos.
For assays which follow aspects of development *in vivo*, we use tissue from different stages of development (in most assays: E14.5, E16.5, E17.5) and perform each assay on tissue from 3 different stages.
For the 6 selected molecules, we have in total 9 different (conditional) mouse models. (For one molecule, we have different cell-type specific conditional ko lines.) For the different mouse lines experiments have already been performed to varying extent. After the careful revision of our data obtained so far and based on our experience in analysing genetically modified mice with a strategy comparable to the one in this proposal, we expect to kill in total a maximum of 700 embryos and corresponding 100 adult pregnant mothers, thus 800 animals in total for these immunohistological experiments.
For histological/ anatomical experiments with tissue from adult mice, we will use both, female and male adult***** animals between P0.5 and 30 weeks. In total, we expect to need here 100 adult animals for all mouse lines together.
For biochemical analysis on neuronal tissue, we will analyze wildtype, heterozygous and knockout/ knockin and expect to need a maximum of 400 animals, estimated 100 embryos and a maximum of 300 adults.
Non-neuronal tissue will only be isolated from mice which are used for the isolation of neuronal tissue.

As a sum, we estimate to kill a maximum of 1300 genetically modified mice for all our *in vivo* experiments; estimated 800 (700+100) embryos and 500 (100+300+100) adult animals. (See supplementary information for more details and the example calculations.)

***** We define for these calculations all post-natal animals (as distinction to the embryonic animals) as "adult", even if they are younger than 8 weeks.

Summary of the estimated numbers:
The total number of **rats is 6500**; with less than 10 % adult, pregnant female animals and more than 90 % embryos.
The total number of wildtype mice is 2840; about 2310 embryos and maximal 530 adult pregnant females.
The estimate for the maximum number of genetically modified mice is round 1875; about 1290 embryos and 584 adults as sum of the 272 (238 embryos (E) and 34 adults (A)) for *in vitro*; 302 (252 E and 50 A) for *ex vivo* and 1300 (800 E and 500 A) for *in vivo* experiments.
Therefore, the **total estimate for all mice is 4715**; about 3600 embryos and maximal 1115 adults.
These animal numbers are estimated based on our experience during the last five years and are in accordance with the new Directive (Directive 2010/63/EU), which also defines in (in contrast to the Directive which was applied so far, also embryos from the last third of the pregnancy as animals.

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: Experiments will be performed *in silico* and *in vitro* as much as possible prior to performing an animal experiment. In case an alternative (i.e. an immortal cell line) is applicable and will answer the research question, these are our first choices. Indeed, we have many different mammalian cell lines in our lab and make extensive use of them, esp. for the motor motility assays or biochemically interaction studies. However, our research question focuses on the specific role of defined molecules in neurons and neuronal development and the majority of the candidate molecules are exclusively expressed in neurons and/ or play a differential role in post-mitotic neurons. Also neuronal cell lines are incompatible with most of our research question due to altered gene expression patterns resulting in the lack or presence of specific molecules. Therefore, even the results obtained in cell lines have to be validated/ proven in primary neurons in most cases as well.
In parallel to our studies in primary neurons and cell lines where suitable, we already work on an alternative strategy which could, in the long-term, replace the use of primary neurons harvested from rat or mice by setting up iPS (induced pluripotent stem) cell cultures and testing protocols to derive cortical neurons from the iPS cells. However, that is long-term strategy and at the moment, neither we nor our collaborators can derive neurons of sufficient quality to answer our research questions. One of the main issues is that iPS cell derived neurons still lack functional synapse formation. We will continue working on this alternative strategy in the next 5 years and stay in close contact with leading labs in this field. Within the next five years, we aim to develop a protocol that allows us to obtain early stage neurons of sufficient quality that can be used to address specific research questions. However, the development of mature neurons with functional dendritic spines is not feasible as a standard technique in the coming 5 years. Therefore, primary neurons harvested from rats or mice are the only suitable and well-accepted model system we can use for the research questions we address with our *in vitro* studies in step 3, 4 and 5 of our project.
Several aspects of neuronal development, specification and function cannot be studied in dissociated primary neurons. Processes like e.g. neuronal migration in the forebrain can only be analyzed within the tissue. To address the role of specific candidate molecules in processes such as neuronal polarization or migration in the cortex, the *ex vivo* approach with tissue obtained from mice described above gives us the unique possibility to discover the function of a gene in these processes by analyzing the consequences of gene expression modifications like knockdown via RNAi, or expression of mutant gene variants. This *ex vivo* approach allows us to reduce the number of animals needed to answer our research questions (see below), however, it depends on neuronal tissue harvested from mice and so far there is no replacement which would allow us to analyze the function of the selected molecules in the mammalian nervous system in a `*like-in-vivo*` situation. In the field of neuroscience, much work is going on to find suitable replacements and for distinct research aspects organoids might be a suitable alternative in the future. However, this technique is currently not sufficiently developed to give reproducible results and to be widely used as model system yet. Therefore, this won't be a possible replacement within the coming 5 years for us.
To also elucidate the physiological role of selected molecules which are suggested to be associated with neuronal malformations/ malfunctions and/or diseases in humans, we use genetically modified mouse models. These *in vivo* model systems give us the unique possibility to discover the physiological function of the selected genes by analysing the consequences of a knockout or knock-in situation in the development, maintenance and function of the CNS within the intact organism. We can address our research questions by histological and anatomical analyses after killing of the animals of different time-points of development. So far, there is no replacement for an animal model which would allow us to analyze the physiological function of the selected molecules in the mammalian nervous system. *Mus musculus* is the smallest animal model system we can choose, which still reflects the human situation to a high extent in the aspects we address with our project. It can be used as model system for research which aims to understand the processes also in the human nervous system and to address human diseases. Moreover, *Mus musculus* is the most widely used model system for research questions like we describe here in our proposal and will allow us to compare and extrapolate our findings and to use them as basis for application oriented research to treat human disease.

Reduction:

In general, all experiments will be executed in succession and despite our extensive experience especially with the experiments in primary rodent neuron cultures and the histological analysis of genetically modified mouse models, small explorative studies will be performed in most cases (referred to as `pilot` or `pre-experiment` above in 2B) to provide the necessary insight in the expected results and their variation. On basis of this explorative work in addition to our experience, the experimental setups and conditions can be optimized for the specific candidate molecule and research question, and statistical analysis can be performed to determine for instance the minimum number of neurons or brains needed for the analysis, and thus, the minimum number of animals needed per experiment to obtain valuable data. To reduce inter- and intra-assay variability, we will use only high-quality and well-established reagents, protocols and technical equipment during the described procedures. However, despite our extensive experience, we constantly aim to refine and improve our techniques and methods, as well as to develop novel and creative methods and experimental designs, which allows us to keep our research state-of-the-art and to obtain high quality research data with a minimum number of animals.

To make optimal use of each animal killed, we take approach-specific measures.

For the *in vitro* approaches, already for several years we successfully use our `neuron-culture-team` strategy in order to harvest the maximum amount of primary neurons and obtain high quality cultures and will continue with this strategy for the next 5 years. With this strategy, the neuron cultures are not prepared by every researcher for his/ her own experiments, but by a `neuron-culture-team`. This team consists of 6 highly skilled researchers from our lab with profound experience and expertise in the preparation of neuronal cultures. They kill the animals, isolate all required brain tissues from the embryos and prepare the neuron cultures from these tissues. Other researchers can then reserve on a list-based system the number of coverslips with neurons they need for their experiments. The main advantages of this system are:

- Very high yield of harvested neurons (reduction: less embryos required)
- High quality standard conditions (reduction: less replications of experiments due to variations in the cultures required; more reliable results)
- No `surplus` embryos; all embryos of one litter are used (reduction)
- Less contamination or other technical issues which could result in the loss of neurons (reduction)
- Animals are handled and killed by very experienced and skilled researchers (refinement)
- No animals just for training of all new researchers in the lab which are using cultured neurons for their experiments required (reduction)

Moreover, other researchers working in other labs on the campus regularly come to collect the non-neuronal tissue of the wildtype rats which we have no purpose for in order to use them for their research.

For most of the experiments in step 3, 4 and 5 where we investigate the candidate molecules in neurons, we use dissociated rat neuron cultures instead of mouse neuron cultures. In this way, we can gain more neuronal tissue from one animal due the bigger size and reduce the number of required animals. The quality of the scientific results is comparable between rat and mouse neuron cultures. Moreover, rat neuron cultures are as widely used as model system as mouse neuron cultures and as such allow the translation and extrapolation of our obtained results.

For the *ex vivo* approaches, we will carefully plan and coordinate all assays for the different 25 candidate molecules we aim to analyse and the specific assays for these molecules in order to reduce the total number of animals. Therefore, we will always use all embryos of a litter and will not have to kill possible `surplus` animals. Since the genetically modification occurs in the majority of the experiments via DNA injection we can define the number of animals used per condition very precisely. As described above, we will first perform several pilot experiments which will need some animals but helps us to reduce the total amount of needed animals due to the standardization and reduction of variance and the possibility to perform more precise power-calculations and therefore reduction of animals afterwards. In general, this method reduces the number of animals needed to address our research question since it can replace the generation of a genetically modified mouse. Moreover, the practical experiments for all

molecule candidates will be performed by a small team of trained researchers to reduce the number of animals needed for training or due to `lost experiments` because of poor quality. Especially for this newly in our lab established technique, we still aim to constantly refine and improve our experimental protocols. For all assays, information about protocol optimization is shared between the lab members and can be used by different researchers where the same question is addressed, just for another selected molecule or similar experiments performed. We thereby avoid using more animals as needed for testing and optimizing experiment conditions and reagents.
Tissue of the wildtype mice which we have no purpose for is offered to other researchers.
To reduce the number of killed genetically modified mice **for the *in vivo* analyses** by histological and anatomical assays, we will carefully plan and coordinate all assays for the specific mouse line, so that we can use the tissue that we harvest from one animal for several different assays. Therefore, we collect both neuronal and non-neuronal tissue from each animal and store the tissue which is not immediately used for later experiments. Wherever possible, this tissue stock is used before killing another animal. Moreover, we collect the cut tissue from the same organ or region of the CNS as discontinuous series on several coverglasses, which can be used for different assays by staining them with different antibody combinations or chemicals. In combination with our strategy to use at least 5 animals, but only from 3 different litters, we ensure with this method to use all available tissue from the region of interest and avoid killing "surplus" animals, due to their genotype which underlays normal biological variation and cannot be predicted or controlled precisely beforehand even if we choose breeding regimes which would result theoretically in a 1:1 ratio of wildtype and knockout offspring. Shared data between the researchers in the lab (online and personally) ensure the coordination of the different assays, even if they are performed by different researchers.

Refinement:
As described above, our teamwork for the rat neuron cultures in the lab ensures that the animals are handled and killed only by very experienced and skilled researchers. For the *ex vivo* approach and the *in vivo* experiments with genetically modified mouse models, one experienced postdoctoral researcher is coordinating the breeding of the lines and assists the other researchers which analyze genetically modified mice with the planning and performance of their experiments. All mice are killed by this postdoctoral researcher or another experienced and skilled researcher in the lab. In this way, we reduce the distress which the animals might experience during the killing, which is the only animal procedure which we perform.

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

The proposed animal procedures described in section 2A are the killing of a rat or a mouse. This will always be performed by skilled and experienced researchers. Cervical dislocation and decapitation are performed within seconds to ensure that pain, fear or suffering is minimal. In accordance with the Directive 2010/63/EU, Annex IV, CO_2 will be used in gradual fill and in all cases the death of the animals is monitored with the highest accuracy and the killing will be completed by additional cervical dislocation.

To avoid discomfort due to the genetic modification of the mice, we will only use breeding regimes which results in animals without discomfort. Like described above in A, we will use conditional knockouts with only cell-type specific depletion of the selected molecule for the lines where the global depletion of the selected molecule would cause discomfort and lethality. Alternatively, for several mouse lines we will use "heterozygous x wildtype" breeding regimes for line maintenance and "heterozygous x heterozygous" breeding regimes only for experiments where exclusively embryonic animals will be analysed (which do not have discomfort). With these strategies, the mice are bred without discomfort and therefore also no additional animal procedure.

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.

Not applicable.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☒ No > Continue with question I.

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

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

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

I. Other aspects compromising the welfare of the animals

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

No other adverse effects are expected. Animals will experience normal housing conditions without additional handling until they are killed.

Explain why these effects may emerge.

Not applicable

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

Not applicable

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.

Indicate the likely incidence.

Expected 0% within time-frame of experiment.

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').

Animals may experience mild discomfort during the procedures described in A.

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 the collection of the tissue necessary for experiments.

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

SUPPLEMENTARY INFORMATION for the estimate of numbers given in B of this appendix (example calculations):

For the *in vitro* approaches:

Example calculations for step 3 (*in vitro* screens), performed in rat primary hippocampal neurons:

Our long-term experience with neuron cultures allows us to estimate the required numbers of neurons per experiment very well. For each molecule we will test, per experiment (N), 1 coverslip with seeded hippocampal neurons will be used and the same for the corresponding controls. With the aim to screen up to 500 candidates in the next 5 years this means 500*4=2000 coverslips with hippocampal neurons. For these screens, we transfect the neurons with the suitable plasmids to drive gene/marker expression and gene suppression of the candidate molecule. For this kind of experiment 100,000 neurons per coverslip are seeded. Thus, 2,000*100.000=200,000,000 neurons are needed for an N=3 plus pilot for all molecules together. With our current isolation protocol, one embryo yields 700,000 hippocampal neurons. Therefore, 200,000,000/700,000=286 embryos are needed for the isolation of primary hippocampal neurons at this step. A normal litter contains approximately 10 embryos. With this estimation 286/10=29 pregnant, adult female rats will be killed. The total amount of animals at this step is therefore: 286 embryos + 29 adult pregnant females = 315 animals.

Example calculations for step 4 & 5 for the *in vitro* analysis, performed in rat primary neurons:

I) For each molecule which we select from the screens, we will first validate the obtained results and the specificity of the effects before we will start with the following investigation of the selected molecule. Therefore, we will perform additional rescue and control experiments in hippocampal neurons and biochemical validation experiments with cortical neurons. Also here, one pilot experiment and subsequent

three experiments under the optimized, constant conditions (N=3) will be performed. For each condition, we will use 2 coverslips with hippocampal neurons per experiment (N) per condition. In general, a proper rescue experiment with all controls contains 4 conditions. Therefore, 4 conditions*2 coverslips=8 coverslips, with N=3+pilot these are 8*4=32 coverslips. We aim to select a maximum of 40 candidate molecules from the screen, thus, 32*40=1280 coverslips are needed. Also here, we will seed 100,000 neurons per coverslip, therefore 1280*100,000=128,000,000 neurons are needed, which will be with 128,000,000/700,000=183 embryos, and with estimated 10 embryos per litter 183/10=18 adult pregnant female rats. In total, we will need for the validation 183 embryos + 18 pregnant mothers = 201, thus around 200 rats. For the biochemical validation experiments, no additional animals are required since we will isolate the cortex from the same embryo used for hippocampus isolation.

II) Functional analysis and nanoscale analysis:

Our experience has shown that per candidate molecule on average 8 different functional assays are performed to elucidate its molecular and cellular role in developing neurons and to unravel the underlying cell molecular mechanisms. For functional assays, several different conditions are tested (e.g. expression of different domains of the protein, interaction studies where several putative interaction partners are tested, different drug concentrations, expression of mutant versions of the protein or specific fusion constructs for motility assays). On average, 7 different conditions (incl. control) are investigated per assay. Per condition normally 2 coverslips are used for these experiments. For all live cell imaging experiments double the number of coverslips with neurons is required due to the phototoxicity of the laser and photo-bleaching during the continuous imaging. Following this estimation, 2 to 4 (on average 3) coverslips*7 conditions=21 coverslips will be needed for one experiment (N). With N=5 (incl. the 2 pre-experiments, thus N=3+2 pilots) these are 21*5=105 coverslips per functional assay for one candidate molecule. With 105*8 assays=840 coverslips with 100,000 neurons each=84,000,000 neurons will be used per candidate for the detailed investigation of the cellular and molecular role of these candidates *in vitro*. We aim to select 40 candidates in 5 years, therefore 40*84,000,000=3,360,000,000 hippocampal neurons are required and since 700,000 neurons per embryo can be isolated, 3,360,000,000/700,000=4800 embryos are needed. Again, we estimate to obtain 10 embryos per litter, therefore 4800/10=480 adult pregnant females will be killed. In total, 4800 embryos + 480 adult pregnant females = concerning these example calculations 5280 rats will be needed for step 4 and 5 of our project.

For the *in vivo* approaches:

For genetically modified mice used for the *in vivo* experiments in step 6:

To investigate the physiological role of the candidate molecules *in vivo*, we will perform immunohistological studies on tissue obtained from genetically modified mice. As explained in A, for the breeding of the genetically modified mice we do not expect any discomfort for the mice since we breed heterozygous animals which don't have discomfort and use the homozygous offspring at embryonic states and therefore before discomfort occurs. Alternatively, we use conditional knockout lines (in which a gene is modified in specific cells or at a specific time) which do not have discomfort. We will mainly use tissue from the central nervous system and normally, the cut tissue from one animal can be collected on 4-6 coverglasses and therefore 4-6 different antibody combinations can be applied. Per scientific question addressed in one assay, we have to stain on average with 3 different combinations. Therefore, tissue from one animal can in most cases be used for two different assays.

Example calculations for experiments with embryonic mice: many of our selected molecules are involved in the development and maintenance of neuronal cells in the CNS and are (suggested to be) associated with developmental as well as degeneration defects or malformation in humans in a disease situation. Our experience shows that we can use embryonic tissue for the *in vivo* analysis of many cell-molecular functions of the candidate molecules. For the assays with embryonic tissue we calculate that one litter contains on average 7 embryos. The tissue from one animal can be mostly collected on 6 glasses. Per experiment we will need 3 glasses for staining, with an N=5 per assay these are 5*3=15 glasses per assay. For assays which follow aspects of development *in vivo*, we use tissue from different stages of development (in most assays: E14.5, E16.5, E17.5) and perform each assay on tissue from 3 different stages, therefore 15*3=45 glasses. Per assay we calculate with 2 glasses for the test of new antibodies,

and on average 3 glasses needed to repeat failed immunostaining, coming to around 50 glasses per research question and assay per genotype, thus 100 glasses for both genotypes. For the profound histological and anatomical analysis of a genetically modified mouse model in our research we calculate based on the experience of the last years, that a maximum of 10 assays are needed. For the 6 selected molecules, we have in total 9 different (conditional) mouse models. (For one molecule, we have different cell-type specific conditional ko lines.) For the different mouse lines experiments have already been performed in varying extent. For 5 lines, we are at the beginning with our *in vivo* analysis with this procedure; for 4 lines, very promising data has already been obtained and several follow-up assays will have to be performed in order to reveal the physiological role of the candidate molecule in the described research interest of this proposal. After the careful revision of our data obtained so far and based on our experience in analysing genetically modified mice with a strategy comparable to the one in this proposal, we expect to kill in total a maximum of 700 embryos and corresponding 100 adult pregnant mothers, thus 800 animals in total for these immunohistological experiments.

Estimations <u>for adult mice based experiments:</u> For several assays will perform histological and anatomical analyses of adult tissue of mice. Again, we will perform an N=5 per genotype per assay and like described above for the embryonic tissue, the collected tissue can in most cases be used for several different research questions by staining with different antibodies or chemicals. For these experiments we will use both, female and male adult****** animals between P0.5 and 30 weeks. In total, we expect to need 100 adult animals for histological and anatomical analyses in this procedure.

For the biochemical analysis, we will perform a maximum of 3 assays (mass spec and/or expression analyses) per mouse line on embryonic and/ or adult****** tissue. Here, one animal per experiment is required. With an N=5 per assay these are 5 animals per assay and 15 per mouse line for each genotype. For biochemical experiment we analyze wildtype, heterozygous and knockout/ knockin. Thus, 15*3=45 animals per mouse line. For all our lines, we expect to kill a maximum of 400 animals, estimated 100 embryos and a maximum of 300 adults for biochemical experiments.

Thus, as sum from the calculations shown above we will kill a maximum of 1300 genetically modified mice for all our *in vivo* procedure; estimated 800 (700+100) embryos and 500 (100+300+100) adult animals.

DEC UTRECHT

A. Algemene gegevens over de procedure

1. Aanvraagnummer : 2017.I.863.016
2. Titel van het project : Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system
3. Titel van de NTS : Het ontrafelen van moleculaire mechanismen die aan de basis van de ontwikkeling en functie van zenuwcellen staan

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

5. Contactgegevens DEC

Naam DEC	: DEC Utrecht
Telefoonnummer contactpersoon	: 088 – 75 59 247
Emailadres contactpersoon	: dec-utrecht@umcutrecht.nl

6. Adviestraject (data dd-mm-jjjj):
   - ☒ ontvangen door DEC: 04-08-2017
   - ☒ aanvraag compleet: 04-08-2017
   - ☒ in vergadering besproken: 16-08-2017
   - ☐ anderszins behandeld:
   - ☒ termijnonderbreking(en) van / tot : 21-08-2017/25-08-2017
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met max. 15 werkdagen:
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD: 12-09-2017

7. De aanvraag is afgestemd met de IvD en deze is hiermee akkoord.

8. Eventueel horen van aanvrager
   - Datum:
   - Plaats:
   - Aantal aanwezige DEC-leden:
   - Aanwezige (namens) aanvrager:
   - Gestelde vragen en verstrekte antwoorden:
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag.

9. Correspondentie met de aanvrager
   - Datum vragen: 21-08-2017
   - Datum antwoord: 25-08-2017

- Gestelde vragen en antwoorden:
  Appendix 1/Bijlage 1:
  - A. Experimentele aanpak en primaire uitkomstparameters: In de tekst onder het kopje 'for the "in vivo approaches"' noemt de onderzoeker het GDL. De DEC raadt u aan dit te verwijderen. *We removed here the GDL Utrecht (page 4).*
  - A. Experimentele aanpak en primaire uitkomstparameters: De verzoekt u om per techniek en per categorie dieren uit te splitsen hoe de dieren gedood worden en waarom dat nodig is voor de experimenten. Dit is nu niet voor alle dieren duidelijk voor de DEC. *We specified now in A, where we "Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach", per technique the category of animals and how the animals of this category will be killed (page 4/5).Moreover, we included in the first sentence of each point why animals of this category are used for the experiments. We kept this as short as possible because the experiment is already described above in A.We also explained in more detail why decapitation is used as the killing method of choice for the embryos of both species, mouse and rat, in the text below the ***. We think, this method is in the case of rodent embryos/neonates in accordance with the Directive, if other methods are not possible and because other methods are not suited for embryos and neonates. Point 12 of the requirements for table 3 of the Annex IV of directive 2010/63/EU specifies decapitation as a possible method if other methods are not possible. (In detail, we explained: "*** Decapitation is used because other methods are not suited for embryos and very young pups. Cervical dislocation by squeezing the neck behind the occiput of the cranium to dislocate the atlanto-occipital joint is not recommended for embryos and neonate pups because they are still too small and the mentioned structures are not sufficiently developed to make cervical dislocation a fast and efficient method of killing. Instead, it increases the possibility that the embryos and neonates suffer. Also, carbon dioxide does not lead to a fast and efficient death of foetal and neonate rodents since they are resistant to hypoxia. CO2 asphyxiation is therefore not recommended for mice embryos and young pups as specified in the requirements of these methods in the table with methods of killing of the Annex IV of directive 2010/63/EU (requirement point 3). Concussion/ percussive blow to the head is also not possible because brain tissue is exactly the tissue we analyze in our experiments (other tissue from embryos is only isolated and analyzed in addition to brain tissue, see also* B*). Finally, injections with an overdose of anesthetics are not efficient in embryos and pups younger than P8 and will cause more discomfort than decapitation. Since all these methods of killing are not suitable for embryos and young pups in our research, the embryos and neonate pups will be killed by decapitation which is listed in the Annex IV of the Directive 2010/63/EU as method of killing for rodents if other options are not possible (requirement point 12 of the table with killing methods).", page 5). We hope that by these changes in the text it now became clear for the DEC how each category of animals is killed, and why.Since we do not expect to analyze pups between P8 and P14, we did not write an extra paragraph for these ages.*
  De DEC verzoekt u ook mee te nemen of het mogelijk/nodig is om bij decapitatie van de foetussen en pups koeling op ijswater (dus niet op alleen maar ijs) toe te passen voorafgaand

aan de decapitatie.)*Thanks for pointing that out. We implement that we will place the embryos on ice-water before decapitation. This a measure which does not interfere with our experiments and outcome parameters. We thought that it is still contradictory discussed if this will cause less discomfort than decapitating immediately, but since this is the field of expertise of the DEC, we are much obliged about this comment from the DEC.*

- D. Vervanging, vermindering en verfijning: Bij verfijning dienen alleen maatregelen genoemd te worden die eraan bijdragen dat het dier minder ongerief heeft. Alle andere maatregelen kunnen verwijderd worden. Het gaat ook niet om verfijning van de techniek. Verfijning van de techniek kan wel tot vermindering leiden, maar dan hoort dat bij het kopje vermindering. *Thank you for this advice. Since indeed most of the refining measures we describe are refinements of the techniques which will reduce the amount of tissue and animals required for experiments, we moved this point to "reduction" and indicated it there by red text (page 11/12). For the point "refinement" we only kept the measure that the animals will be handled and killed exclusively by very experienced and skilled researchers to reduce the distress which the animals might experience during the killing, which is the only animal procedure which we perform (page 13)*

.- L. Wijze van doden: Voor een deel van de dieren wordt afgeweken van de richtlijn. Dit dient nog aangevinkt en gerechtvaardigd te worden (zie ook de vraag over A). *Like we explained now in detail in A, we still think that we kill all animals, including the embryos (and neonates) in accordance with the Annex IV of directive 2010/63/EU. In L it is explicitly asked, "Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?" Since decapitation is listed in the table 3 of the Annex IV of Directive 2010/63/EU as possible method for rodents if other methods are not possible (point 12 of the requirements which specify the table) and we argued now in A in detail why other methods are not possible, we think that no changes in L are required any more. We think that we should give our explanation for the decapitation of embryos/neonates in point L instead of A, since we thought in L should be only given an explanation if it is not listed in the Annex. We are looking forward to the response and advise of the DEC regarding our argumentation.*

Also, the implementation to place the embryos/pups on ice-water is included in A instead of L since that is not part of the Annex IV of Directive 2010/63/EU. We hope that it became clear with our explanation in A, why we filled L with a "yes"

- NTS (Niet Technische Samenvatting):- 3.2 Opbrengsten project en wetenschappelijke en/of maatschappelijke belang: Het is niet helder wat bedoeld wordt met 'overschrijven'. Graag verduidelijken of herformuleren. *Thank you for this advice, we agree with the DEC and replaced "overschrijven" by "herstellen" (page 2). This word better reflects our purpose, which is to rescue or regain the normal and functional situation.*

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

10. Eventuele adviezen door experts (niet lid van de DEC)
    - Aard expertise:
    - Deskundigheid expert:

- Datum verzoek:
- Strekking van het verzoek:
- Datum expert advies:
- Advies expert:

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er zijn geen DEC-leden betrokken bij het betreffende project.

C. Beoordeling (inhoud):

1. Toetsbaar en voldoende samenhangend?

Afwijkingen in intracellulair transport worden beschouwd als kritische factoren bij de ontwikkeling en degeneratie van neuronen in zowel het centrale als het perifere zenuwstelsel. Morfologische en functionele afwijkingen van het cytoskelet en disfunctionerend axonaal transport worden beschouwd als een van de eerste en kritische pathogene verschijnselen in neurodegeneratieve ziekten zoals Alzheimer, ALS en SMA. Onderbreking van axonale microtubili en/of axonaal transport resulteert in verstoring van vescicle transport , verandert specifieke interacties tussen getransporteerde moleculen en veroorzaakt defecten in retrograde signalen, hetgeen uiteindelijk leidt tot het afsterven van neuronen en het verlies van neurale functies zowel centraal als perifeer. Diverse recente studies beschrijven mutaties in microtubilaire motoreiwitten en hun bindingseiwitten in de mens, direct gerelateerd aan pathologieën van motorneuronen (b.v. SMA) en afwijkingen in de corticale ontwikkeling. Ondanks toenemende aanwijzingen voor een relatie tussen verstoorde neurale ontwikkeling en een aantal neurodegeneratieve ziekten enerzijds en defecten in actine- en microtubulair-afhankelijk transport anderzijds, ontbreekt nog veel mechanistische kennis met betrekking tot de organisatie van het cytoskelet, axonaal transport en hoe dit transport wordt aangetast in geval van ziekte of functieverlies, dan wel overexpressie van bepaalde genen. De nog gebrekkige kennis van deze cellulaire mechanismen is een van de belangrijkste redenen waarom effectieve behandeling van neurodegeneratieve ziekten voorlopig onmogelijk is.
De overall doelstelling van dit project is, via fundamenteel onderzoek, te komen tot meer (genetische) kennis en begrip van het belang van het cytoskelet en intraneuronale transportprocessen. Belangrijke moleculaire componenten van deze processen zullen worden geïdentificeerd in zowel normale neuronen, als wel in neuronen die zijn gemodificeerd ten aanzien van specifieke genen. De te verwachte resultaten zullen bijdragen aan de kennis met betrekking tot ontwikkeling en functioneren, maar ook met betrekking tot degeneratie van neuronen, en vormen de basiskennis voor het ontwikkelen van toekomstige, effectieve therapieën om neurodegeneratie te voorkomen en/of te genezen.

Uit de projectbeschrijving blijkt naar de mening van de DEC-Utrecht dat het gaat om een samenhangend project met een duidelijke focus en een overzichtelijke onderzoekstrategie. De DEC kent een substantieel belang toe aan de vraagstelling en het geschetste onderzoeksplan.
De DEC-Utrecht is van mening dat het een toetsbaar project is, het meest overeenkomend met voorbeeld 1 uit de handreiking.

2. Voor zover de DEC bekend, is er geen mogelijk tegenstrijdige wetgeving die het uitvoeren van de dierexperimenten in de weg zou kunnen staan.

3. De in de aanvraag aangekruiste doelcategorie(ën) sluit(en) aan bij de hoofddoelstelling(en).

Belangen en waarden

4. Het directe doel van het project is, via fundamenteel onderzoek, te komen tot meer kennis en begrip van het belang van het cytoskelet en intraneuronale transportprocessen. Belangrijke moleculaire componenten van deze processen zullen worden geïdentificeerd in zowel normale neuronen, als wel in neuronen die zijn gemodificeerd ten aanzien van specifieke genen.
Het uiteindelijke doel van het project is bij te dragen aan de kennis met betrekking tot ontwikkeling en functioneren, maar ook degeneratie van neuronen, en het leggen van de basis voor toekomstige, effectieve therapieën om neurodegeneratie te voorkomen en/of te genezen. Het gaat hier om belangrijk fundamenteel onderzoek. Of, en hoe, de resultaten ervan uiteindelijk hun toepassing zullen vinden kan nu nog slechts in algemene termen worden weergegeven.
Desondanks is de DEC van mening dat er in voldoende mate een relatie is tussen het directe doel en het mogelijke uiteindelijke doel. Het directe doel is gerechtvaardigd in de context van het onderzoeksveld.

5. De belangrijkste belanghebbenden in dit onderzoeksproject zijn: de (toekomstige) patiënten, de proefdieren en de onderzoekers. De eerste belanghebbenden zijn de (toekomstige) patiënten. Neurodegeneratieve ziekten hebben een grote, negatieve impact voor de patiënt zelf, maar ook voor zijn/haar omgeving en de maatschappij als geheel. Het mogelijk voorkomen en/of genezen van dergelijke ziekten is van groot belang. Voor de proefdieren geldt dat zij zullen worden gedood om weefsels te verkrijgen voor ex vivo onderzoek. De dieren hebben er belang bij hiervan gevrijwaard te blijven. De dieren zullen in het kader van het onderzoek gedood worden. Voor de onderzoekers geldt dat dit project kan bijdragen aan een goede wetenschappelijke reputatie en kan leiden tot nieuwe wetenschappelijke inzichten. Wetenschappelijke reputatie kan door de onderzoeker van belang geacht worden, maar dient naar de mening van de DEC geen rol te spelen in de ethische afweging over de toelaatbaarheid van het gebruik van proefdieren.

6. De aanvrager geeft niet aan nadelige effecten op het milieu te verwachten. De DEC ziet geen aanleiding om aan te nemen dat zich toch nadelige effecten zullen voordoen.

Proefopzet en haalbaarheid

7. De kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven zijn voldoende gewaarborgd en dragen eraan bij dat de doelstellingen behaald kunnen worden, dat aan de 3V-beginselen voldaan kan worden en dat voorkomen kan worden dat mens, dier en milieu negatieve effecten ondervinden als gevolg van de dierproeven.

8. Het project is zeer systematisch opgezet, de voorgestelde experimentele opzet en uitkomstparameters sluiten logisch en helder aan bij de aangegeven doelstellingen en de gekozen strategie en experimentele aanpak zal zeker leiden tot het behalen van de fundamentele wetenschappelijke doelstelling binnen het kader van het project. De onderzoekstrategie volgt een 7-stappenplan. In de stappen 1 en 2 zijn dmv massaspectrometrie, *in silico* analyse en literatuurstudie ongeveer 500 genen geïdentificeerd, die mogelijk van belang zijn voor verder onderzoek aan intraneuronaal transport via het cytoskelet. In de stappen 3-5 wordt m.b.v. *in vitro* onderzoek met primair gekweekte neuronen het aantal genen die in aanmerking komen voor verder onderzoek sterk teruggebracht. Pas in de stappen 6 en 7 worden de proefdieren gebruikt voor *ex vivo* en *in vivo* onderzoek voor het karakteriseren van uiteindelijk slechts 6 genen. De figuren 1 en 2 en de bijbehorende tekst onder 3.4, 3.4.1 en 3.4.2 geven een helder inzicht in de opeenvolgende stappen voor deze strategie en de go-no go momenten voor de meeste genen met de argumentatie daarvoor.

Welzijn dieren

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

10. De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de EU richtlijn.

11. Het cumulatieve ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Volwassen dieren worden gedood met CO2, gevolgd door cervicale dislocatie. De pups worden

gedood door decapitatie, voorafgegaan door plaatsing op ijswater. Beide methoden leiden tot licht ongerief. Genetische modificatie leidt in geen enkel geval tot constitutioneel ongerief.

12. De aantasting van integriteit bestaat uit het doden van de dieren en het gebruiken van hun hersenweefsel voor verder onderzoek. Hoewel het doden van de dieren op zichzelf gezien kan worden al een aantasting van hun integriteit, is de DEC van mening dat de aantasting van de integriteit gekwalificeerd zou moeten worden als licht, uitsluitend in verband met het feit dat de dieren instrumenteel gebruikt worden voor dit onderzoek.

13. Humane eindpunten als gevolg van de experimenten zullen in dit project niet worden bereikt.

3V's

14. Vervanging: De aanvrager heeft voldoende aannemelijk gemaakt dat voor een deel van het project er geschikte vervangingsalternatieven zijn. Zo zijn de stappen 1 en 2 uit het project uitgevoerd middels massaspectrometrie , *in silico* analyse en literatuurstudie. In het project zijn hiervoor verder geen proefdieren aangevraagd. De DEC is er van overtuigd dat de aanvrager pas overgaat tot onderzoek met weefsels van dieren die voor dat doel gedood zijn op het moment dat alle mogelijkheden om zonder dieren een verdere selectie te maken uitgeput zijn.

15. Vermindering: Het aantal te gebruiken dieren is realistisch ingeschat en er is een heldere strategie om ervoor te zorgen dat tijdens het project met het kleinst mogelijke aantal dieren wordt gewerkt waarmee nog een betrouwbaar resultaat kan worden verkregen. De stappen 3-5 worden uitgevoerd met primaire kweken van neuronen. Hoewel hiervoor ook aanzienlijke aantallen dieren nodig zijn betekent dit toch een relatief sterke vermindering. De onderzoekers geven een uitgebreide en overtuigende onderbouwing van het aantal benodigde proefdieren, hoofdzakelijk gebaseerd op de hoeveelheid cellen die nodig zijn voor de opeenvolgende stappen in het onderzoek.

16. Verfijning: Het doden van volwassen dieren vindt steeds plaats onder CO2 verdoving, gevolgd door cervicale dislocatie. Embryo's worden gedood door decapitatie, voorafgegaan door plaatsing op ijswater. Het project kent geen experimentele handelingen waarop verfijning van toepassing zou kunnen zijn voorafgaand aan het doden van de dieren. Het project is daarmee in overeenstemming met de vereiste van verfijning van dierproeven en het project is zodanig opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd.

17. Er is geen sprake van wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Dieren van beide geslachten zullen in gelijke mate worden ingezet.
Daar waar de proefdieren embryo's betreft wordt gebruik gemaakt van beide geslachten. De volwassen dieren die de embryo's leveren zijn uiteraard vrouwelijk. Daar waar volwassen muizen

worden gebruikt voor *in vivo* onderzoek zullen zowel mannetjes als vrouwtjes worden gebruikt, maar de resultaten worden sekse-specifiek geanalyseerd.

19. De dieren worden in het kader van het project gedood. Het onderzoek in alle stappen in het project wordt uitgevoerd aan primaire kweken van neuronen, dan wel aan delen van geïsoleerde hersenen. Daarvoor worden de betreffende embryo's en volwassen dieren gedood. De moederdieren die de embryo's leveren worden noodzakelijkerwijs ook gedood. De dieren worden in overeenstemming met bijlage IV van de EU richtlijn, op een passende wijze gedood.

20. De vraag over hergebruik is niet van toepassing omdat de dieren gedood worden in het kader van het experiment.

NTS

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

D. Ethische afweging

1. De morele vraag waarvoor de DEC-Utrecht zich gesteld ziet is of het belang van het onderzoek naar genen die essentieel zijn voor het functioneren van het cytoskelet en voor een goed verloop van alle aspecten van axonaal neuronaal transport, en waar bij verstoring van die genen mogelijkerwijs leidt tot het ontstaan van neuronale degeneratie/ziekten, de onvermijdelijke aantasting van het welzijn en de integriteit van de gebruikte proefdieren kan rechtvaardigen.

2. Er vindt een beperkte aantasting van welzijn en integriteit van de proefdieren plaats, met licht ongerief.
   Indien de doelstellingen behaald worden, dan zal dit project bijdragen tot een toename van de fundamentele kennis van bouw en functie van het cytoskelet in neuronen, van het axonale transportsysteem en van de genen die coderen voor eiwitten die hier een essentiële rol bij spelen. Verstoring van deze processen, genetisch dan wel anderszins, liggen waarschijnlijk ten grondslag aan het ontstaan van neurodegeneratieve ziekten zoals Parkinson, Alzheimer en ALS. Een beter begrip van deze neuronale processen en de verstoring daarvan kan de basis vormen voor de ontwikkeling van effectieve therapieën ter voorkoming en genezing van neurodegeneratieve ziekten. Het is aannemelijk dat de fundamentele doelstellingen van het project behaald zullen worden. Daarvoor is de inzet van proefdieren noodzakelijk, maar de onderzoekers doen al het mogelijke om het ongerief voor de dieren en het aantal dieren tot een minimum te beperken. Dat het voor de individuele onderzoeker van belang kan zijn om aansprekende onderzoeksresultaten te boeken is juist, maar in de uiteindelijke afweging kent de DEC-Utrecht daar weinig gewicht aan toe.

3. Op grond van het bovenstaande is de DEC van oordeel dat het vergaren van fundamentele kennis en begrip van axonale transportprocessen via elementen van het cytoskelet, met het oog op het begrijpen van het ontstaan en verloop van neurodegeneratieve ziekten een substantieel belang vertegenwoordigt en dat dit substantiële belang opweegt tegen de beperkte aantasting van het welzijn en de integriteit van de proefdieren. Het gebruik van de proefdieren zoals beschreven in de aanvraag is daarmee gerechtvaardigd.

E. Advies

1. Advies aan de CCD

   X 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...

   ☐ De DEC adviseert de vergunning niet te verlenen vanwege:
   - ☐ De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:...
   - ☐ De volgende doorslaggevende ethische bezwaren:...
   - ☐ De volgende tekortkomingen in de aanvraag:...

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn geen knelpunten/dilemma's naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies.

Centrale Commissie Dierproeven

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Universiteit Utrecht
[redacted]
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3501 AA UTRECHT

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0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD1080020173404
Bijlagen
2

Datum 18 september 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 18 september 2017. Het gaat om uw project "Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD1080020173404. 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:
18 september 2017
Aanvraagnummer:
AVD1080020173404

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
18 september 2017
Aanvraagnummer:
AVD1080020173404

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10800
Naam instelling of organisatie:	Universiteit Utrecht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	30275924
Postbus:	12007
Postcode en plaats:	3501 AA UTRECHT
IBAN:	NL27INGB0000425267
Tenaamstelling van het rekeningnummer:	Universiteit Utrecht

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

Datum:
18 september 2017
Aanvraagnummer:
AVD1080020173404

Gegevens plaatsvervangende verantwoordelijke onderzoeker

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

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 januari 2018
Geplande einddatum:	1 januari 2023
Titel project:	Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system
Titel niet-technische samenvatting:	Het ontrafelen van moleculaire mechanismen aan de basis van de ontwikkeling en functie van zenuwcellen
Naam DEC:	DEC Utrecht
Postadres DEC:	Postbus 85500 3508 GA Utrecht
E-mailadres DEC:	dec-utrecht@umcutrecht.nl

Betaalgegevens

De leges bedragen:	€ 1035,-
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:	Utrecht
Datum:	18 september 2017

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD1080020173404
Bijlagen
2

Datum 18 september 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 18 september 2017
Vervaldatum: 18 oktober 2017
Factuurnummer: 173404
Ordernummer: o.v.v. CB.841910.3.01.011

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD1080020173404	€ 1035,00

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.

From: info@zbo-ccd.nl
To: Instantie voor Dierenwelzijn Utrecht
Cc: [redacted]
Subject: Aanhouden AVD1080020173404
Date: woensdag 18 oktober 2017 13:40:07

Geachte [redacted]

Op 18-09-2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system" met aanvraagnummer AVD1080020173404. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Niet technische samenvatting
In de NTS geeft aan hoeveel volwassen dieren worden gebruikt tijdens het project. Dit aantal komt niet overeen met het aantal volwassen dieren in de aanvraag. U wordt verzocht dit aan te passen. Daarnaast geeft u bij vraag 3.4. van de NTS aan dat er geen negatieve gevolgen zijn voor het welzijn van de dieren. De dieren worden echter gedood. U wordt verzocht dit ook aan te passen.

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

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

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

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

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

[redacted]
www.centralecommissiedierproeven.nl

.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl

Beste CCD,

Bedankt voor het zorgvuldig doornemen van onze projectaanvraag.

U vraagt ons het aantal volwassen dieren in de NTS aan te passen, omdat het niet overeen zou komen met het aantal in de appendix van onze aanvraag. We hebben dit punt nog een keer nauwlettend gecontroleerd, en zijn er nog steeds van overtuigd dat de aantallen in beide documenten juist zijn en overeenkomen. Het aantal in de NTS omvat alle volwassen dieren, dus muizen en ratten. (Dus: 584 volwassen genetisch gemodificeerde muizen + 530 volwassen wildtypische muizen + minder dan 650 volwassen ratten = in totaal maximaal 1750 volwassen dieren.) Desalniettemin zijn we dankbaar voor de opmerking dat dit niet helemaal duidelijk wordt in de NTS, en we hebben daarom nu ook in de NTS onderscheid gemaakt tussen muizen en ratten bij de volwassen dieren.

Bovendien vraagt u ons punt 3.4 van de NTS aan te passen. We hebben ook dit punt aangepast, en schrijven nu dat we geen negatieve gevolgen voor het welzijn verwachten tot de dieren worden gedood en dat het doden het enige ongerief voor de dieren is.

Verder hebben we geen veranderingen in de NTS aangebracht.

We hopen dat de aanpassingen aan de NTS voldoende duidelijkheid verschaffen en de vragen van de CCD afdoende hebben beantwoord.

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven

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Universiteit Utrecht
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Onze referentie
Aanvraagnummer
AVD1080020173404
Bijlagen
1

Datum 22 november 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 18 september 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system" met aanvraagnummer AVD1080020173404. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a lid 1 van de Wet op de dierproeven (hierna: de wet).

U kunt met uw project starten. De vergunning wordt afgegeven van 1 januari 2018 tot en met 31 december 2022. Deze termijn is anders dan in uw aanvraag, omdat een vergunning niet voor langer dan vijf jaar mag worden afgegeven.
De onderbouwing van deze beslissing vindt u onder 'Overwegingen'.

Procedure

Advies dierexperimentencommissie
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie (DEC) DEC Utrecht gevoegd. Dit advies is ontvangen op 18 september 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Het advies van de DEC is betrokken bij de behandeling van uw aanvraag.

Nadere vragen aanvrager
Op 18 oktober 2017 hebben wij u om aanvullingen gevraagd. U heeft antwoord gegeven. De aanvullingen hadden betrekking op de NTS. Uw antwoord is betrokken bij de behandeling van uw aanvraag.

Overwegingen
Alle hierboven genoemde stukken liggen ten grondslag aan ons besluit.

Wij kunnen ons vinden in de inhoud van het advies van de DEC, inclusief de daaraan ten grondslag liggende motivering.

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).

Datum:
22 november 2017
Aanvraagnummer:
AVD1080020173404

Centrale Commissie Dierproeven
namens deze:

[redacted]
Algemeen Secretaris

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

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Universiteit Utrecht
Adres:	Postbus 12007
Postcode en plaats:	3501 AA UTRECHT
Deelnemersnummer:	10800

deze projectvergunning voor het tijdvak 1 januari 2018 tot en met 31 december 2022, voor het project "Identification of the molecular mechanisms underlying cytoskeleton dynamics, cytoskeleton based transport and synapse organization in neurons of the central nervous system" met aanvraagnummer AVD1080020173404, volgens advies van Dierexperimentencommissie DEC Utrecht.
De functie van de verantwoordelijk onderzoeker is Hoogleraar.
Het besluit is gebaseerd op de volgende (aangepaste) stukken:

1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 18 september 2017
2 de bij het aanvraagformulier behorende bijlagen:
   a Projectvoorstel, zoals ontvangen op 18 september 2017;
   b Bijlagen dierproeven
      - 3.4.4.1. Elucidation of fundamental cell and molecular mechanisms underlying cytoskeleton dynamics, transport, synapse organization and neuronal specification and migration in rodent neurons in vitro, in organotypical brain slice cultures ex vivo and in vivo by histological/ anatomical analysis of genetically modified mice models., zoals ontvangen op 18 september 2017;
   c Niet-technische Samenvatting van het project, zoals ontvangen op 18 september 2017;
   d Advies van Dierexperimentencommissie zoals ontvangen op 18 september 2017
   e De aanvullingen op uw aanvraag, ontvangen op 23 oktober 2017.

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Overige opmerkingen
3.4.4.1. Elucidation of fundamental cell and molecular mechanisms underlying cytoskeleton dynamics, transport, synapse organization and neuronal specification and migration in rodent neurons in vitro, in organotypical brain slice cultures ex vivo and in vivo by histological/ anatomical analysis of genetically modified mice models.				Ratten: 5850 embryo's en 650 volwassen dieren Muizen: 3600 embryo's en 1115 volwassen dieren
	Ratten (Rattus norvegicus)	6.500	100,0% Licht	
	Muizen (Mus musculus)	4.715	100,0% Licht	

Aanvraagnummer:
AVD1080020173404

Ter informatie
Onderstaande informatie is opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.

- Go/ no go momenten worden voor aanvang van elk experiment afgestemd met de IvD.
- Het is verboden een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is.
- Het is verboden dierproeven te verrichten voor een doel waarvan het belang niet opweegt tegen het ongerief dat aan het proefdier wordt berokkend.
- Overige wettelijke bepalingen blijven van kracht.

Aanvraagnummer:
AVD1080020173404

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.