		Inventaris Wob-verzoek W17-08								
			wordt verstrekt				**weigeringsgronden**			
nr.		**document NTS 2017808**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
1		Aanvraagformulier				x		x	x	
2		NTS oud			x					
3		Projectvoorstel oud				x			x	
4		Bijlage beschrijving dierproeven 1 oud				x			x	
5		Bijlage beschrijving dierproeven 2 oud				x			x	
6		Bijlage beschrijving dierproeven 3 oud				x			x	
7		Bijlage beschrijving dierproeven 4 oud				x			x	
8		DEC-advies				x		x	x	
9		Ontvangstbevestiging				x		x	x	
10		Verzoek om aanvullende informatie				x		x	x	
11		Reactie verzoek aanvulling 1				x		x	x	
12		Reactie verzoek aanvulling 2				x		x	x	
13		NTS aangepast	x							
14		Bijlage beschrijving dierproeven 1 nieuw				x			x	
15		Bijlage beschrijving dierproeven 2 nieuw				x			x	
16		Bijlage beschrijving dierproeven 3 nieuw				x			x	
17		Bijlage beschrijving dierproeven 4 nieuw				x			x	
18		Advies CCD		x						x
19		Beschikking en vergunning				x		x	x	

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* 10700
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	Universiteit Maastricht
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	50169181

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

Straat en huisnummer	Minderbroedersberg 4-6
Postbus	616
Postcode en plaats	6200 MD Maastricht
IBAN	NL04 INGB 0679 5101 68
Tenaamstelling van het rekeningnummer	Universiteit Maastricht

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]	☐ Dhr. ☒ Mw.
Functie	Associate Professor	
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	PhD Student	
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	01 - 02- 2017
		Einddatum	01 - 02- 2022
3.2	Wat is de titel van het project?	Autoimmunity of the nervous system	
3.3	Wat is de titel van de niet-technische samenvatting?	Autoimmuniteit van het zenuwstelsel	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC-UM
		Postadres	Postbus 616([redacted] 6200 MD Maastricht
		E-mailadres	[redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1584 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	[redacted]
Plaats	Maastricht
Datum	2 - 1 - 2017
Handtekening	[redacted]

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	Autoimmuniteit van het zenuwstelsel
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Autoimmuniteit, zenuwstelsel, myastenia gravis, immunisatie, immuun-therapie

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)

Recent is bewijs gevonden dat auto-immuun antistoffen ook syndromen van het centraal zenuwstelsel kunnen veroorzaken zoals schizofrenie. Hierbij kunnen auto-antilichamen bijvoorbeeld niet alleen spieren of gewrichten aantasten, maar ook neurologische en neuropsychiatrische symptomen veroorzaken. De ziektemechanismen van auto-antilichamen in het zenuwstelsel zijn echter nog niet goed bekend. Dit project zal deze auto-antilichamen van het zenuwstelsel karakteriseren in patiënten, nieuwe doelwitten identificeren en nieuwe behandelingen voor deze patiënten testen.

3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Patiënten met neurologische of psychiatrische aandoeningen zullen door betere identificatie van deze antilichamen beter behandeld kunnen worden. Verder verwachten we dat door identificatie van nog onbekende auto-antilichamen meer kennis over auto-immuun ziekten verkregen wordt hetgeen een adequate behandeling ten goede komt. Voor sommige auto-immuunziekten zijn er nu zelfs helemaal geen behandelingen beschikbaar. Concreet worden in dit project nieuwe diagnostische testmethodes als basis voor nieuwe therapieën ontwikkeld.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Voor dit project zullen muizen, ratten en konijnen gebruikt worden. Wij schatten maximaal 5128 dieren, waarvan 3294 ratten, 1744 muizen, en 90 konijnen nodig te hebben over een periode van 5 jaar. g
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	De dieren kunnen bijwerkingen krijgen van de te testen medicatie en hiervan stress ondervinden; de chirurgische ingrepen kunnen eveneens stress veroorzaken. Daarnaast zullen de dieren ongerief ondervinden van de immunisatieprocedure alsmede van de symptomen/effecten van auto-immuunziekten. Ook van de gebruikte gedragstesten zullen de dieren ongrief ondervinden.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Zoals beschreven in de bijlagen ondervinden alle dieren ongerief van mild tot matig, dit door gedrags, neurologische en bewegingstesten, de immunisatie met Complete Freud's Adjuvant (appendix 2) en het doorsnijden van de zenuw aan een poot zorgt voor matig ongerief. Omdat de stoornis gedurende de tijd ontwikkelt kan het, eventuele, ernstige ongerief vroegtijdig geobserveerd worden. Wanneer het ongerief vordert naar ernstig zullen de dieren de humane eindpunten bereiken en opgeofferd worden. Hierdoor zullen de dieren geen ernstig ongerief ondervinden.
3.6	Wat is de bestemming van de dieren na afloop?	Na afloop van het project zullen de dieren worden geëuthanaseerd waarna het weefsel wordt geanalyseerd.

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.	Er zijn nog geen alternatieve modellen beschikbaar die complex genoeg zijn om de interactie tussen alle factoren (o.a. zenuwstelsel en immuunsysteem) te bestuderen. Daarnaast is het nodig om klinische effecten aan te tonen en de bijwerkingen te bestuderen, voordat een nieuw medicijn toegepast kan worden op de mens. Indien er alternatieve methodes beschikbaar zijn (zoals hybridoma cellijnen of expressievectoren) voor de productie van antistoffen zullen wij hiervan gebruik maken.

4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Statistische methodes worden toegepast om zo weinig mogelijk dieren te gebruiken. Tussentijdse analyses worden uitgevoerd om het aantal proefdieren zo klein mogelijk te houden. Go/no-go momenten zijn op verschillende momenten ingepland voor het vermijden van onnodige experimenten.
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.	De diermodellen voor myasthenia gravis, een autoimmuun ziekte waarbij auto-antilichamen receptoren aanvallen in de neuromusculaire junctie met voornaamste resultaat een verzwakt en vermoeid ziektebeeld, zijn goed gevalideerd als auto-immuun ziektemodel en voor het testen van medicijnen voor deze ziekte. De onderzoekers hebben veel ervaring in het werken met dit model en de gebruikte methodes. Andere diermodellen zullen nieuw ontwikkeld worden. Afhankelijk van verwachte symptomen/phenotype/parameters zullen wij de meest geschikte diersoort/species kiezen. Konijnen worden alleen gebruikt voor het opwekken van antilichamen. Dit diermodel is het meest gebruikt omdat het immuunsysteem van konijnen voordelen heeft ten opzichte van dat van knaagdieren. De immuunreactie geeft een breder spectrum en meer specifieke antistoffen.
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	Er wordt pijnstilling en anesthesie gebruikt om ongerief te verminderen. Indien dieren meer ongerief vertonen dan verwacht, wordt het experiment voortijdig beëindigd.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
1.3	Provide the title of the project.	Autoimmunity of the 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.

Autoimmune diseases are caused from the over activity of the immune system against molecules and tissues normally present in the body. In autoimmune diseases, the immune system and genetic and environmental factors play all an important role in the pathologies. There are several hypotheses that try

to explain the origin of autoimmunity. Clonal anergy, idotype network, clonal ignorance, suppressor population and clonal deletion are more prominent.

Autoimmunity affects 7-9 % of the general population (Cooper et al., 2009) and its clinical spectrum varies from organ specific to systemic diseases. A large number of diseases have been discovered targeting the nervous system inducing a wide variety of syndromes ranging from musculoskeletal to neurological, and neuropsychiatric. Targets/antigens of the nervous system are mostly voltage gated ion channels, ligand gated receptors or proteins associated to these membrane antigens. It is not surprising that an antibody response to this kind of targets has far reaching effects on the functioning of the neurons ([redacted] et al, 2013). Autoimmune neuromuscular channelopathies (primarily Myasthenia gravis (MG); one of most well-known autoimmune disorders, where the autoantibodies target molecules presents in the neuromuscular junction, such as acetylcholine receptor, resulting in a weakness and fatigability clinical symptomatology. There are many animal models that reproduce the human disorder by active or passive immunization and which have been used to describe the targets, study the pathogenic mechanisms and define the efficiency of new therapies to treat the disease) have been well studied and are exemplary for the study of pathological mechanisms of autoantibodies in the peripheral (PNS) and central nervous system (CNS). Complement deposition and inflammation, modulation of the receptor function, antigen internalization and loss or block of the receptor associated proteins are the pathogenic mechanisms described in many nervous system autoimmune disorders. However, the pathways underlying more recently identified disorders still remain unknown. Increasing the knowledge in this area will help the clinicians to understand the pathologies and test and provide more efficient treatments to the patients, who in some cases don't have the chance to be treated yet.

Recently, pertinent evidence has been published suggesting an important role for auto-antibodies in a number of CNS syndromes. The cerebrospinal fluid (CSF) always contains antibodies present in the periphery (Poduslo et al, 1994). There is a large body of evidence that auto-antibodies (e.g. in autoimmune channelopathies) reach sites in the CNS and have immunopathogenic effects (Diamond et al, 2009; [redacted] et al, 2013) in spite of the protection posed by the blood-brain-barrier (BBB). At the same time intrathecal antibody production, meaning the presence of antibody producing B-cells in the CNS, was detected in patients with N-methyl-D-aspartate receptor (NMDAr) encephalitis (Martinez-Hernandez et al, 2013; Endres et al, 2015). Since 2005, every year 1 or 2 novel antigens of CNS and their associated syndromes were discovered (Leypoldt et al, 2014). The methodology for the detection of these autoantibodies is based on diagnostic assays using rat antigenic tissue. Most neuronal ion-channels and receptors fulfil essential functions and are highly conserved between species. These findings changed important paradigms within autoimmunity and also neuropsychiatric diseases and still the pathogenic mechanism of many of the recently discovered autoantibodies are not well understood. Noticeably, all these disorders targeting membrane proteins react well to immunosuppressive therapy. Of special attention to us is also the role of these autoantibodies in neuropsychiatric disorders. The pathological mechanisms of disorders such as schizophrenia and bipolar disorder are poorly understood, but likely this diagnosis is a wide collection of patients with different pathological mechanisms. The hypothesis of antibody mediated autoimmunity as cause in schizophrenia has gained increasing attention as it was supported by several findings (Pandarakalam, JP., 2013; [redacted] P. et al., 2015; [redacted], C. et al., 2016): (a) comorbidity of neuropsychiatric diseases and autoimmune diseases, (b) susceptibility genes increasing risk for neuropsychiatric disorders are involved in immune regulation, (c) cytokine dysregulation, (d) association with infectious diseases and (e) responsiveness to immunosuppressive therapy.

[redacted]

[redacted]

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 project focuses on 3 main aims (Figure 1):

1. Generation of antigenic and antibody sources (see appendix 1): they can be used for the induction of the animal model and/or the assessment of the development and progression of the animal model. It is important to mention that the proteins obtained from this point will also be used for the screening of the presence of autoantibodies, in patients with disorders related with the nervous system and with a potential autoimmune disorder origin.

   To this end, we will use animals to produce antigen rich tissue from adult animals and embryonic cells of interest (mostly primary neuronal cells) (appendix 1-1) which can then be used to test reactivity of human blood or CSF to these tissues. This is possible, since neuronal receptors and ion-channels have a high inter-species homology (Lancaster E., et al., 2010, Meizan L, et al., 2009). We will use rats for brain extraction, female pregnant rats for embryonic primary cell cultures (appendix 1-1) and male rats to obtain AChR after transection (Rochkind S. and Shainberg A., 2013) (appendix 1-3).

   Additionally, immunization of animals with the antigen of interest will be included under this purpose since the antibodies (appendix 1-2) generated will be used for the induction of a passive transfer autoimmune animal model and/or to assess the development and the progression of the animal models. Only antibodies that are not commercially available or cannot be generated without the use of animals will be generated under this license. We will use rabbits to generate polyclonal antibodies and mice, to generate monoclonal antibodies. In both cases, there is a preference on female, since they are reported to be group housed more successfully than males because females are more docile and less aggressive in social interaction (Hendriksen and Hau, 2003).

2. Generation of nervous system autoimmune animal models (see appendix 2 and 3): by active and passive immunization to develop known and new models and to characterize the pathogenicity, effector mechanisms and spectrum of symptoms of antibodies targeting the nervous system. The antigens or antibody molecules to induce the model are not all derived from this project proposal, since sometimes they can be obtained recombinantly, like the antibodies cloned from a patient or obtained from a company, like the AChR from *Torpedo californica*).

   To answer this question, we will perform active or passive immunization in rodents (rats and mice) to develop models of autoimmune diseases of the nervous system and study their behavioural and pathological phenotypes and the mechanisms used by the antibodies against an

specific target to generate the disease.

The use of knock-out (KO) animal models will not answer the question of the antibodies role in neurologic and/or psychiatric disorders, either the description of their pathological mechanisms, since the KO approach will only cover the blocking of the target protein, but it is known that autoantibodies can have other mechanisms of action like, activation, internalization, activation of the complement, etc.

The selection of the gender will depend on the available literature and the standardized guidelines available for each model. For example, the active immunization model for myasthenia gravis or EAMG is standardized by using Lewis female rats. However, there are other models where the gender is not robustly stablished. An example of it is that, in the literature we have found described no differences between male and female mice in some of the strains, such as C57Bl6, one of the most common strains used in experiments related with our research (Papenfuss T. L., et al., Journal of Neuorimmunology, 2004). This data is in correlation with some studies where mice have been injected with patient autoantibodies, where in some cases males where chosen (Hammer c. Et al., Molecular Psychiatry, 2014; Planaguma J. Et al., Brain, 2015) as female (Castillo-Gomez R. Et al., Annals of Neurology, 2016). Because of this, we would like to keep open the decision on the mice gender, since we will adapt it to the most recent literature before starting the experiment (see project proposal, section 3.2, second aim).

The procedure is similar to aim 1 (generation of antibodies by active immunization) with the difference that animals will develop the full-blown pathology, since the exposition time to the antigen is longer than when animal models are developed. Additionally, the strain and species used in antibody production can differ to those used for autoimmune animal models and therefore react differently to the immunization.

We are interested in neuropsychiatric disorders with an autoimmune origin. We have based the selection of our antibodies and its pathogenic target or antigens and the correspondent animal models in the literature. Since the NMDAr has been described as a cause of autoimmune encephalitis, many other neuronal receptors have been identified to be the target of autoantibodies in other patients causing similar phenotypes. This means that other antigens and its autoantibodies will be included in case of newly discovered as a cause of an autoimmune neuropsychiatric disorder.

3. New therapies efficacy studies in nervous system autoimmune animal models (see appendix 4): new and repurposed drugs will be assessed using relevant models previously generated in aim 2 (rats and mice) to characterize autoimmune treatments and asses their side effects.

These 3 goals can be met in a period of 5 years, because we can start at aim 1, 2 and 3 simultaneously for different antigens currently in different research stages. The best characterized disease of MG can directly be used to study drug effectiveness and side-effects, whereas NMDAr encephalitis has been characterized in preliminary mouse models (Planagumà, J. et al., 2014; Hammer, C. et al., 2013) and will thus be further characterized according to aim (2) and then (3). [redacted]

3.3 Relevance

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

By screening populations of neurologic and psychiatric disorders for the presence of autoantibodies on diagnostic assays developed from animal tissue, we aim to identify a subgroup of patients with an autoimmune origin of their symptoms. Analysis of autoantibodies will provide a proof of concept in case the disease symptoms are caused by a specific targeted autoimmune condition and decipher the pathogenic mechanisms behind the condition. This will give insight into which neurotransmitter signalling/ neuronal pathways and/or cell types are affected in disease and thereby provide a better understanding of the disease mechanisms. This will directly reveal therapeutic targets in the concrete autoimmune condition but can also bring to light some general knowledge on which neuronal pathways are affected in disorders like schizophrenia. Then, new or repurposed drugs can be used as alternative therapies for these pathologies which are more efficient and induce less aggressive/less side effects. For example, specifically in myasthenia gravis, some patients who do not improve by using the current available treatments will benefit from the study of new therapies targeting other pathways.

Clinicians will be able to diagnose and treat patients in a more efficient manner. This would be of strong impact on a subgroup of patients since many patients with neuropsychiatric disorders are diagnosed early in their life and will be strongly impaired chronically. A targeted treatment would have a great impact for these patients and disburden the psychiatric hospitals and institutions.

3.4 Research strategy

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

We will describe in the following section the different strategies to reach the 3 main research goals:

1. Production of antigenic and antibodies molecules for the generation and/or characterization of the development and progression of known or new nervous system autoimmune animals models and screening and identification of novel autoantibodies targeting molecules in the nervous system in human diseases.

    a) Adult rat tissue isolation: brains will be isolated to perform an immunohistochemistry (IHC) analysis for the detection of autoimmunity in patients with autoimmune disorders affecting the nervous system. We will use material from neurologic and psychiatric sick patients coming from in house samples, samples from other hospitals or from new studies already approved by the ethical committee. Also, the tissue will be used as a tool to identify novel antigens through an immunoprecipitation technique. Muscles from denervated rats will be also used to isolate AChR which will be used to describe the development and progression of the animal model pathology using techniques like ELISA or radioimmunoassay (RIA) to identify autoantibody levels in animal models and patient's material.

    b) [redacted]

    c) [redacted]

[redacted]

2. Autoimmune animal model generation targeting neuronal surface proteins or molecules expressed in the NMJ, using active or passive immunization strategies. Novel and known models will be induced and characterized by studying the spectrum of symptoms generated by the antibody action in each case.

   Rodents will be actively or passively immunized to develop models of autoimmune diseases of the nervous system and study their behavioral, neurological and locomotion characteristics and the pathology. The up/downregulation or expression of specific genes/proteins involved in the nervous system will be used to study the role of these proteins in naïve animal and animal models where nervous system pathology is induced to describe the specific role of those proteins in these disorders.

3. Drug efficacy and side effects in nervous system animal models.

[redacted]

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

[redacted]

A further description of the procedures performed in each level is explained:

1. Production of antigenic tissue and antibody molecules for the generation and/or characterization of nervous system autoimmune animal models and study of novel autoantibodies targeting the nervous system in human diseases.

   In appendix 1 we describe the creation of antigenic tissue and antibodies for different purposes

including the generation of primary cell cultures, material used for screening (AChR for RIA and brain for IHC), or the antibodies required to generate passive transfer models. Within this appendix, no animal will undergo any procedure as behavioural, neurological or locomotion tests. It will be animals of different ages (embryonic to adult) which will be sacrificed for isolation of tissue containing the antigen of interest. The mothers will be sacrificed and their tissue will also be used to store antigen containing tissues (mainly the brain) and embryos will be isolated for primary cell culture (Appendix 1). For the generation of AChR the tissue will be enriched by performing a sciatic nerve transection and letting the animals recover which will increase the number of synapses (sprouting) and thereby the density of AChR in the tissue (Appendix 1). The generation of antibodies in vivo in rodents will be also performed under this aim since the antibodies obtained from the active immunization of rodents can in turn be used for the development of autoimmune animals models generated by passive transfer and/or the characterization and screening of autoantibody levels in animal models and patient's material.

2. Development and characterization of novel or known nervous system autoimmune disorders.

   Rodents will be immunized actively (with antigen) (Appendix 2) or passively (with autoantibodies) (Appendix 3) which will induce an autoimmune diseases targeting neuronal surface antigens or molecules expressed in the NMJ. Depending on the antigen, we expect different symptoms which we will measure with a defined set of behavioural, neurologic and locomotion tests. In the end of the experiments animals will be sacrificed and tissues collected for analysis of pathogenic mechanisms.

3. Efficacy and side effects study of new or repurposed drugs to treat autoimmune diseases of the nervous system.

   In appendix 4 we describe the procedures for testing treatments in the animal models described before. The animals will thus perform similar behavioural, neurological and locomotion examinations as in appendix 2 and 3, but will be treated with new and/or repurposed drugs with special focus on drugs targeting the immune system or protecting the target organ (i.e. targeting plasma cell: proteasome inhibitors ([redacted], [redacted]. et al., 2010, and protein silencing; [redacted] et al., 2009, [redacted] et al., 2016). Again, the animals will be sacrificed in the end of all procedures and their tissue analysed for signs of inflammation etc.

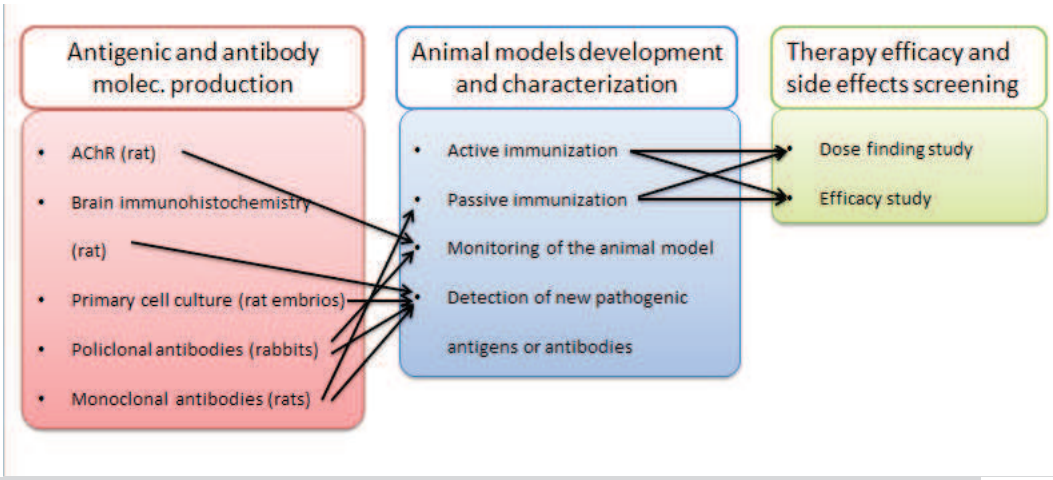

Figure 1. Correlation between the outcomes of each aim proposed in this protocol.

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

The different components of the described project are largely interlinked with the common objective to describe and treat nervous system disorders of autoimmune origin. Well known and novel antigens of the CNS or the PNS and antibodies targeting these antigens relevant to human disorders will be produced (appendix 1) and used to generate animal models (appendix 2 and 3) which will be used to test efficient treatments and its side effects (appendix 4). Novel pathogenic antibodies will be identified using antigenic sources generated in appendix 1. Antigens and antibodies will be used to generate and characterize the development and progression of the disorder in the animal models. The animal models will be characterized in detail for those antigens/antibodies that have not been described previously. The animals will be injected with the antigen (active immunization) or with the autoantibodies that causes the disease (passive immunization) available in house, in some cases the antibodies will be obtained from recombinant patient autoantibodies expressed in cells for example or the antigens from another source like the electric organ of the *Torpedo californica* in case of the AChR. Animal models will be characterized *in vivo*, using behaviour, neurologic and locomotor tasks and also in an *in vitro* level, to ensure the progression of the pathology through the alteration of the immune system markers and to describe the pathological mechanisms underlying each disorder. Later on, once the model is robustly stablished, potential therapies to treat the diseases will be screened using these animal models. The drugs tested will vary from standard, repurpose to novel.

Following the flow chart from Figure 2 those are the go/no go criteria for the study in general, more specific criteria will be specified in the specific appendix concerning the question.

a) Pathogenic molecules source available: go criteria to the production of the antigenic and antibody molecules in case of no quality available material from an external source (no -go criteria).
b) Quality and quantity: the material obtained from the generation of antigenic molecules will be tested for quality and quantity, in case it will not follow the requirements a no go criteria for the next step will be applied and a new antigenic molecule will be generated changing some variables in the experiments (e.g. species, gender, route of administration, frequency, dose, adjuvants, etc.). Once the antigenic product gave the results expected in terms of quality and quantity, a go

criteria will be followed to generate autoimmune animal models. An example of quality is the immunogenic capacity of the antigen, the conformation, the affinity and other parameters that will be tested in vitro. The amount required for each purpose varies.

c) Robust and standard: the capacity to reproduce the animal model and the mimicry with the human disorder will be taken into account. A no go criteria will be determined by a not strong enough difference between the animal model and its control or because of non-reproducible data between the different experiments. Since the animal models concerning CNS and autoimmunity are not still well stablish by anyone, we don't know which phenotype we can expect. Because of this, we will follow the procedures performed by well-known laboratories which already performed similar experiments to characterize in the past other autoantibodies and their targets. If no differences are observed between the model and its control, we will modify some parameters (concentration, administration via, frequency, etc.) to see whether the effect of the antigen/ antibodies can be shown. When the model shows a robust phenotype and pathogenic mechanisms are observed, a go criteria for the next step, therapy efficacy and side effects screening, will be followed. An example of standardization and reproducibility is the presence of autoimmunity in the animal model (minimal significant difference of 10% between the groups in behaviour, neurological and locomotion tasks and detectable levels of immunoglobulins, complement system molecules, cytokines or other inflammatory markers and the capacity to reproduce these changes in different experiments following the same procedures.

   After this point one of the main objectives is reached: the generation and characterization of a novel nervous system autoimmune animal model.

d) Efficient and safe: the success of a new therapy in an animal model will be described and also the side effects. The experiment will proceed if the animals develop autoimmunity as described in c. A no-go criteria will be considered in case the therapy did not show any improvement in the animal model compared with the control. Next the dose and/or the route of administration will be modified. In case the side effects are tolerable, considering the specific human endpoints (>20% weight loss, severe acute inflammation at the immunization point and self-induced trauma), we will have a go criterion. If an improvement is observed, with a reduction of the disease phenotype and the reduction of pathogenicity a go criteria will be followed using this drug in other autoimmune model.

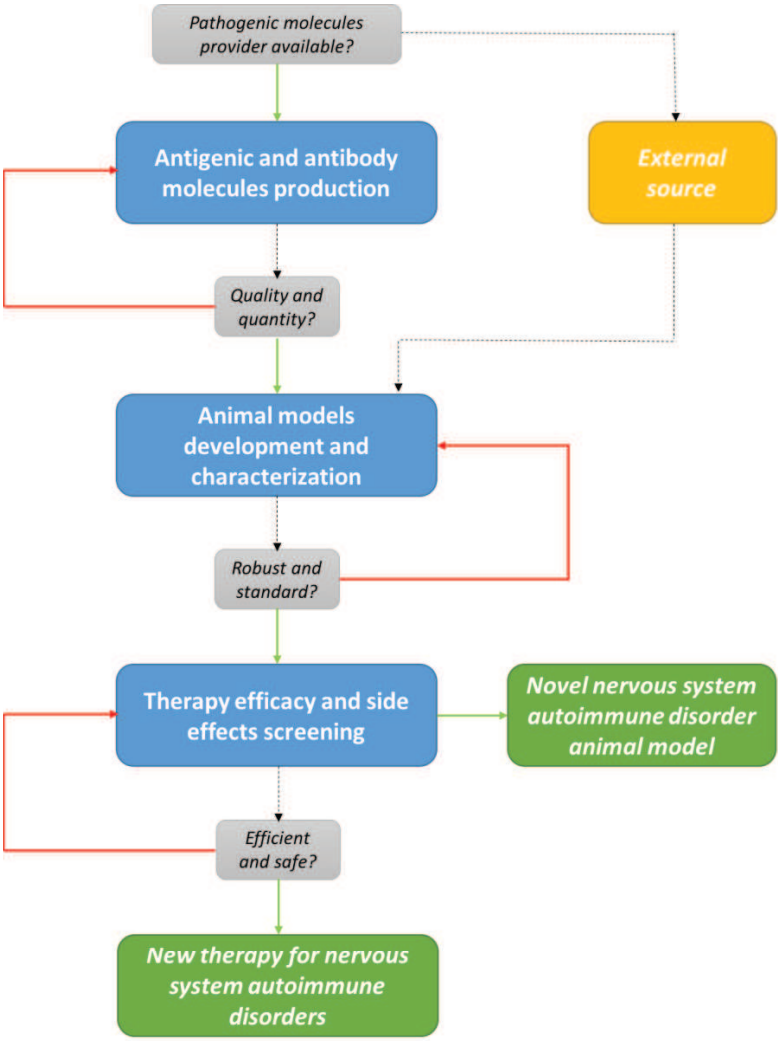

Figure 2. Go and no-go criteria flow chart for the study. The criteria to take into account are expressed in the grey boxes, the main steps of the study are contained in the blue boxes and the objectives showed in green boxes. Green arrows became a go criteria while red arrows mean no-go criteria.

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

Serial number	Type of animal procedure
1	Generation of antigenic and antibody sources (1. Brain tissue insolation)
2	Generation of antigenic and antibody sources (2. Antibody generation)
3	Generation of antigenic and antibody sources (3. Acetilcholine receptor extraction)
4	Active immunization in rodents
5	Passive immunization in rodents
6	Therapy efficacy and side effect in nervous system autoimmune models
7	
8	
9	
10	

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
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: 1 Type of animal procedure: Generation of antigenic and antibody sources

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.

This appendix describes the creation and isolation of antigen and antibody rich material using animals to generate and follow the progression of the animal models and the drug screening on them. The same material will be used to identify novel pathogenic antigens in patient's material, which is a positive feedback process to generate novel animal models to describe the pathogenic mechanisms underlying the disease and try therapeutic approaches to treat them. This will include (1) the production of primary cell cultures of neurons, astrocytes and microglia, (2) isolation of tissue of expected location of antigen, which is brain, peripheral nerves and muscle tissue and (3) generation of antibodies by immunization to use them on the development of passive models.

The primary outcome of this section is based, on one hand, on the generation of diagnostic assays to improve the detection of antibody mediated autoimmunity in central nervous system (CNS), generating rat embryonic primary neuronal cell lines and rat adult brain slices for fluorescent or normal staining, two techniques already well stablished as a diagnostic method in patients with neurologic and psychiatric impairment. Neuronal antigens are expressed in high densities in the brain when compare with other tissues in the body and have a high interspecies homology (Table 1), so all known autoantibodies causing CNS diseases in humans cross-react with the rat antigen (Lancaster E., et al., 2010, Meizan L, et al., 2009). For this purpose, pregnant female rats and the embryos/pups will be recovered by caesarean section between day 18 and birth. The mother will be sacrificed in any case and the brain isolated for use as described above.

On the other hand, the generation of acetylcholine receptor (AChR) is included in this project using a denervation technique, where big amounts of the antigens will be generated and then isolated (Rochkind S. and Shainberg A., 2013). The protein obtained can be used to perform assays where antibodies from patients or other animal models can be analysed and to generate the active immunization models for AChR (Appendix 2).

For the generation of *in vivo* antibodies, we will follow the code of practice for the immunization of laboratory animals. Weekly injections of the antigen of interest are performed, *(s.c.)* to the animal until the immunoglobulin levels reach the peak of expression, around 2-6 weeks after the first injection. The best time point will be determined by testing IgG levels in the blood of the animals (obtained from saphenous vein, every 3-10 days maximum, following the NC3R guidelines for blood sampling).

The use of strong adjuvants (like Complete Freund's Adjuvant, CFA) is required to break tolerance to self-antigens (autoimmunity models by active immunization, see appendix 2). However, for general antibody production (as required here), the use of alternative adjuvants (like incomplete Freund's adjuvant, IFA) will be enough since for this purpose autoimmune reaction is not required. Later on, the animal will be sacrificed and material (e.g. serum and/or splenocytes) will be used for the induction of a passive transfer autoimmune animal model (see appendix 3) and/or to assess the development and the progression of the animal models (see appendix 2 and 3).

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

For the neuronal primary cell cultures the animals will be sacrificed (by decapitation) between embryonic day 18 (retrieved from the uterine horn) and just after birth to create primary cell lines of different cell phenotype. The age depends on the antigen of interest and the expression profile during the certain age. The cells will be used for a diagnostic test to analyse whether autoantibodies against brain antigens are present in serum or cerebrospinal fluid (CSF) of patients with potential central nervous system (CNS) autoimmunity or autoimmune rodent models. Additionally, these cells will be used for *in vitro* assays to study the pathogenic effect of patient autoantibodies on neuronal cells, e.g. internalization of antigen and alteration in ion channel function. Primary cell culture will preferentially be obtained from the unused pups coming from the breeding of the other animal procedures described in this proposal. We are currently working on the setting up of the embryonic cell culture assay, using 1 day born animals, since some groups in the university are regularly working with these animals and we could take the brain as a surplus material.

Rats will be sacrificed to isolate the brain and tissue from other organs (e.g. liver, spleen, etc.) to use as control. The tissue is used for immunohistochemistry (IHC) with patient's IgG in serum or CSF as primary antibody and immunoprecipitation of human autoantibodies. This method is common for the detection of autoantibodies against neuronal antigens in patients, since the homology between the human and the rat proteins is higher than 92% (Lancaster E. 2010; Meizan L. et al., 2009) (Table 1). It is preferred over the use of human post mortem material for different reasons: (a) The antigens are spread over the whole brain, so the complete brain has to be used for the method, but usually a limited amount of serum/CSF is available to do the staining, which leads to practical difficulties on human brain tissue. (b) The fixation and freezing of the tissue has to be done in a very consistent way to guarantee trustful, replicable diagnostic assays; human tissue is commonly fixed with formaldehyde or freshly frozen, which is not the optimal preparation of the tissue for our purpose. (c) Additionally, the post mortem delay significantly affects the sensitive antigens and introduces variability that that might lead to misdiagnosis (false positive or false negative) of autoantibody presence. The animals used for this purpose will mostly be the mothers of the embryos used for creation of primary cell cultures. Yet, during times in which no experiments with embryos are necessary, females from breeding, and males will be also used for the

purpose of brain isolation. In case no embryos are required at this period and a denervation experiment is ongoing, brain from the denervated males will be also used for this purpose since no gender differences have been identified. It is important to mention that when no primary cell cultures are necessary, brains from rats euthanized in experiments from other researchers at the university will be used in case they are available.

Table 1. [redacted]

[redacted]	[redacted]	[redacted]	[redacted]
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[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

For the antigen enriched tissue generation of AChR generation, male rats will have a sciatic nerve transection in one of the hind legs. We will use 9 week old (275g) male rats, since those specimens are bigger and the amount of muscle is more abundant due to hormonal differences. In female rats the effect of estrogen on the nervous system would induce a variation that would request higher number of animals per group. Animals will undergo a partial sciatic nerve transection under complete anesthesia in one of the hind legs (unilateral) to reduce the discomfort. Peri and post-operative analgesia will be applied for min 3 days post-operative, prolongation on indication. After the intervention, the nerve will start recovering by sprouting and new neuromuscular junctions will be created, with a higher expression of AChR (Rochkind S. and Shainberg A., 2013). Two weeks later, animals will be brought under anaesthesia and sacrificed. Afterwards, the receptor derived from rats will be isolated and used in multiple experiments in appendixes 1, 2 and 3. The active immunization models for AChR-MG (immunized using *Torpedo californica* AChR (tAChR)) results in an antibody production targeting not only the tAChR, but also the rat AChR. The amount of antibodies in the serum is a good indicator for the severity of disease in these animals. However, in order to be able to measure the concentration of antibodies against the AChR in the serum of rodents, a radioimmuno assay (RIA) needs to be carried out, wherein the rodent AChR is a necessary reagent. To this end, the animals of this appendix will be euthanized and the carcasses will be then used to extract the antigen.

The methods are operational for some antigens e.g. AChR. For those neuronal antigens that are yet to be identified, the protocol might have to be changed, e.g. the animal might need to be perfused for optimal preparation of the tissue.

For the generation of antibodies in animals, rabbits and rodents will be immunized with the antigen of interest. Animals will be tested for raising immunoglobulin levels in serum to determine the number of antigen injections and to sacrifice the animals at the highest peak of antibody levels. Animals will not follow any other kind of tasks and control animals are not required for this purpose. If any of the animals develop a phenotype, as a result of the immunization, the standard humane endpoints (section J) will be applied. For this purpose, the preservation or the homology between the proteins in human and rabbit/rodent is not necessary, since the animal will only develop antibodies against the antigen injected, and we will determine the animal species in advance based on our specific interest. The antibodies

generated, in this case polyclonal antibodies, will be used to label the organs where the antigens are expressed, to see, for example, if the injection of autoantibodies reduces the density of that specific protein. On the other hand, monoclonal antibodies can be used for the purpose mentioned before but also to generate passive transfer animal models (Kreye J., et al. Brain, 2016).

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

The number of animals is based on the literature and on previous experience (Elmariah SB et al., 2005, Dalmau J. et al., 2008). As described below we will also make use of rest material of other rodent studies at Maastricht University to minimize the number of animals.

B. The animals

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

Rats will be used for this experiment, based on previous experience. Wistar female pregnant rats is the most suitable strain for primary cell culture and brain tissue material based on the literature (however no strain and gender changes has been observed using brains for IHC in our group), while Lewis male rats are the most convenient for sciatic nerve transection due to the biggest size (directly proportional to the muscle and AChR) (e.g. 9 week old Wistar male rat with 275g weight aprox.).

We estimate to use one pregnant rat (~10 embryos/pups) per month, so in a period of 5 years: 60 mothers.
Animals will only be sacrificed upon need. According to the patient material available, one or two animals at a time will be sacrificed. It is known that 1 animal serves around 100 usable brain slides, which will each allow testing one human sample for antibody presence. We expect to need around 2 animals per month, which means 120 rats over a period of 5 years. However, we are currently already performing this method with tissue from other department of the University which is giving sufficient supply for our current experiments and we already have a biobank of around 15 brains for this purpose, so numbers are likely to be much lower (or even zero) if other departments continue doing similar experiments.

For the denervation experiment, an estimated number of 18 adult male Lewis rats will be used based on previous experience. We use adult rats, because we want an antibody repertoire of the adult tissue. In the other appendixes included in this proposal, we will need AChR for about 1500 antibody determinations/experiment with a maximum of 3 repetitions. For each determination, it is necessary to have 150 µl of receptor in solution, which means a total of 675 ml of AChR receptor solution (1500*3*0.150=675ml). In the RIA the standard curve corrects for the concentration of antigen. From an adult rat, 37.5 g of muscle tissue is suited for receptor isolation and taking into account the conversion factor of 600 ml receptor suspension/500g muscle, only 45 ml of receptor solution can be obtained from each individual (600 ml/(500 g/37.5 g/rat)=45 ml/rat). Based on these values, 15 animals will be required to provide enough antigen for the assays proposed. However, experiments in the past have shown that there is a failure rate of 15 % in animals undergoing sciatic nerve transection surgery. Therefore, three additional animals are necessary. In total, we use a max. of 18 male rats.

For polyclonal antibody generation, animals with a strong immune response are recommended taking

into account also that the material obtained from them will be larger. Because of this, female rabbits are the best option for large quantities. Alternatively, we will use rodents because this is the target animal for passive transfer models. We have planned to generate antibodies against a maximum of 6 different antigens and for each we will use a maximum of 15 animals (based on previous experience, which means maximally 90 animals for this purpose). Additionally, we will use mice for monoclonal antibody generation. In that case, we will use maximal 10 animals per antigen (6 antigens), so a total of 60 mice.

A total (maximum) of 348 animals (198 rats, 90 rabbits, and 60 mice) are included in this appendix. All animals will be obtained from a registered supplier. These numbers

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.

Concerning refinement, the animals will be housed in groups and with enriched cages to allow species typical behavior. We do not expect any discomfort as the animals will not undergo any procedures in the primary neuronal cell culture and brain slices but we expect moderate discomfort in the sciatic nerve resection. For antibody production, refinement will take place by using anaesthesia and analgesia for the invasive procedures which minimize the discomfort of the animals.
Concerning generation of antigenic tissue, replacement by e.g. cell lines is not possible, because cancer cell lines express many antigens that create background staining of the human antibodies and thereby false positive results.

- For antigen specific tests, some expression systems for e.g. NMDAr, AMPAr, LGI1, and Caspr2 are available and we are using those.
- No cell line exists which overexpresses the receptors/ion channels (complex membrane channel, composed of 4 or more subunits) for this purpose taking into account international guidelines.
- For brain material source, if no embryonic primary cell lines are required, we will ask for rats of other animal studies or we will use, in case a denervation experiment is running, the brains from those animals. Human brain tissue is not an option to use in IHC, since the amount of tissue we can access is low, the quality and fixation of post-mortem human tissue is not of sufficient quality and the size of the human brain is too large, requiring an unrealistic amount of human serum to be tested.

Concerning the production of antibodies, we will only make use of animals in case there is no hybridoma or expression vector available for producing the antibody targeting the antigen of interest.

- Rabbits are the most suitable specie to generate antibodies *in vivo* but as a replacement Lewis female rat are also a good option based on their enhanced immune response described when generating active immunization models.

Reduction will be considered by using surplus animals when available for the isolation of brains and also the brains from the pregnant female and the rat males used for AChR insolation. For the primary cell culture of neuronal tissue, we are currently trying to set up the technique using 1 day postnatal animals since some groups are regularly working with these animals in the university and they don't need the brain.

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

The well-being of animals will be checked daily and in case of severe suffering or disease (not expected) animals will be sacrificed according to the human endpoints.

Repetition and duplication

E. Repetition

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

The tissue will be used to characterize autoantibodies in patient serum or CSF or to determine antibody presence in autoimmune models described in all the other appendices. We are analysing sera and CSF of individuals that have not been characterized before. This is assured by communicating with the clincial center that provides the patient material. To our knowledge from conferences and literature there are still many autoantibodies to neuronal antigens still unknown of which the function is not studied.

Accommodation and care

F. Accommodation and care

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

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

The animals will be anesthetized during the nerve transection. Additionally, we will use pain killers pre and post-operatively. For antibody production, analgesia will be administered in case of discomfort on the injection site. Optimal procedures will be discussed and decided on with the local IvD/AWB when writing the working protocols.

I. Other aspects compromising the welfare of the animals

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

Due to nerve transection we expect locomotion impairments and possibly pain development of the wound after the surgery. From immunization with antigenic molecules, we expect the development of normal autoimmune reaction including inflammation and starting symptoms of the disease. From the use of adjuvants we expect pain in the injection area and an exacerbated immune reaction.

Explain why these effects may emerge.

The only discomfort could arise from complications in the pregnancy and sciatic nerve transection. The transection can impair movement of the hind limbs or paralysis for 48 hours after surgery. For the last issue, analgesia will be administered to the animal if required. Post-operative (also after immunization) analgesia will be applied for min. 3 days post-operative, prolongation upon indication.

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

Animals will be monitored daily. If any adverse reactions occur (not expected) the experiment will be halted. The discomfort generated by the nerve transection will be diminished with the use of pain killers pre and post-surgery. If animals have problems feeding, we will make use of liquid food or extra rich food to increase the weight or keep it constant. We will also make sure that the animals are able to reach food and water at all times. The discomfort generated by antigen immunization will be covered with analgesics. Humane endpoints will be applied.

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.

Humane endpoints will be applied in case of rapid weight loss (>20% within 3 days without any improvement), severe acute inflammation at the immunization point (untreatable by the definition of the veterinarian) and in case of self-mutation or autotomy after nerve transection. It is important to mention that according to our experience, none of the animals followed these behavior, even receiving a bilateral transection.

The following enpoints are unlikely, but for completeness we include them since some phenotypes have never been tested before. These include prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).

Indicate the likely incidence.

We expect 0-0.5% of cases will require humane endpoints. We do not expect any problem from the pregnant rats to obtain primary cell cultures and brain tissue. For denervated animals, animals can suffer discomfort due to the paralysis and stress related. For *in vitro* autoantibody generation we do not expect any severe symptomatology since the duration of the experiment will not allow the development of it.

K. Classification of severity of procedures

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

The procedure is a non-recovery procedure for neuronal tissue generation and organ collection. In the case of sciatic nerve denervation, the discomfort is moderate. The discomfort generated by the nerve transection will be diminished with the use of pain killers pre/post-surgery. Later on the discomfort is based on the paralysis of the hind legs. The animals will be able to move crawling 48h post-surgery and they will be sacrificed after a period of 2 weeks. The discomfort related with the antibody production will be moderate, due to the immunization with incomplete Freund adjuvant or similar and the animals will only suffer an immune reaction with a mild expression of the disease).

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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.

It is necessary to sacrifice the animals to extract cells to start primary cell culture or extract antigenic and antibody tissue.

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

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

☒ Yes

Appendix
Description animal procedures

a) This appendix should be enclosed with the project proposal for animal procedures.
b) A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
c) For more information, see our website (www.centralecommissiedierproeven.nl).
d) 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'.	10700
1.2	Provide the name of the licenced establishment.	University Maastricht
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: 2 — Type of animal procedure: Active immunization in rodents

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.

This appendix describes the development of active immunization rodent models for autoimmune mediated diseases that target antigens in the nervous system. Depending on the antigen that is used to immunize the animals, different nervous system pathologies can be developed. We intend to immunize with neuronal receptors and ion channels leading to autoimmune encephalitis including N-methyl-D-aspartate receptor (NMDAr) and other receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AMPAr and B gamma-aminobutyric acid receptor; GABABr) or channel associated proteins such as Leucine-rich glioma inactivated 1 (LGI1) or contactin associated protein 2 (Caspr2) which are associated to the voltage gated potassium channel (VGKC), but also antigens in the peripheral nervous system (PNS), expressed in the neuromuscular junction (NMJ), which includes the acetylcholine receptor (AChR), muscarinic kinase receptor (MuSK) and LDL receptor related protein 4 (Lrp4). The injection of any of these antigens (generated in appendix 1 using animals or commercial or recombinant origin) is used to produce an animal model of the disease and/or to study the pathologic progression/mechanisms of the disease and the symptoms induced in the animals. For example, it has been proven in myasthenia gravis (MG), where the injection of MuSK or AChR develop MG and are already well established animal models for autoimmunity (experimental autoimmune myasthenia gravis or EAMG). For central nervous system (CNS) antigens, active immunization has been performed in some pathologies like Multiple sclerosis (MS) where myelin proteins are injected to develop an inflammation model called experimental autoimmune encephalomyelitis (EAE) (Denic A. et al., 2013; Mix E. et al., 2010). This give direct evidence and supports the hypothesis that the injection of the antigen will develop the disorder in an animal model. An autoimmune disease can be either T-cell or B-cell mediated (or a combination of both). To determine what the driving pathogenic force is, the immunized animals will be used to isolate antibodies, T-cell and B-cells and each fraction can subsequently be transferred to naïve animals (passive transfer) to test pathogenicity (which is described in

Appendix 3).

The antigen will be obtained from one of the following sources, depending on the state of research:

a) External sources: electric organ of the *Torpedo californica*, recombinant autoantibodies, *in vitro* systems based on the production of the antigen of interest using eukaryotic cell lines (recombinant DNA in prokaryotic and eukaryotic systems) or any other antigenic SOURCE used to immunize and generate a nervous system autoimmune animal model of interest.
b) In house generation: as described in Appendix 1, the antigenic and antibody molecules have to be, in some cases, in house generated because they are not commercially available. AChR will be obtained from denervated muscle rat (since *in vitro* methods do not produce enough antigen for this purpose), brain expressed antigens will be obtained from rat brains and primary cell cultures from rat embryos and in vitro generated antibodies will be obtained by antigen immunization in rodents. Some of the material will be also used to screen patients' plasma/sera or CSF to identify new pathogenic antigens (e.g. rat brain tissue for immunohistochemistry using patient sera).

The antigen will be administered peripherally (sub cutaneous (s.c.)) to the animal.

The primary outcome is the generation and characterization of autoimmune models targeting the nervous system and will include behavioral (symptomatic), neurological and locomotor tests, followed by post mortem analysis to describe the production of antibodies, T-cells and B-cells and the pathogenic mechanisms of autoantibodies.

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

The immunization varies on the animal model as described above. The frequency is also determined by several parameters like the immunogenicity and stability of the antigen, the severity of the induced disease, etc. with a minimum of a unique immunization at the beginning to a weekly injection until the end of the experiment. Additionally to the antigen, we will use immunogenic reagents to exacerbate the immune reaction since a strong immune response and a break in the tolerance to self-antigens are required to generate an autoimmune model. For this purpose we will use Complete Freund's Adjuvant (CFA) which will be mix with the antigen of interest in an emulsion, injected s.c. in 4 different places in the base of the tail (incomplete FA (IFA) will be used in case of repetitive immunizations (Li, 2004)). CFA will be used to generate the animal models following international guidelines for reproducible and reliable autoimmune models in MG ([redacted] et al., 2015) and other models like collagen-induced arthritis (Brand DD. et al., 2007), autoimmune uveitis (Bansal S. et al., 2015), Tourette syndrome animal model (Horning M. and Lipkin W.I., 2010), experimental autoimmune encephalitis (Randohoff R.M., 2012; Denic A. et al., 2013) or experimental allergic encephalomyelitis (Pachner A.R., 2011) among others. The duration of the experiment varies between 6 to 9 weeks.

a) For the development of a disease, animal models will be generated by a single immunization or weekly immunizations as a maximum frequency, in the same way but studied for up to 9 weeks (chronic model) in case of repetitive immunizations the first immunization will be performed using CFA and the following/s using incomplete FA (IFA) (Li, 2004). During this time, animals will perform different behavioral and neurological tests as described below and will be sacrificed at different time points to analyze pathogenicity and signs of inflammation. Also the blood (obtained from saphenous vein, every 3-10 days maximum, following the NC3R guidelines for blood sampling) will be analyzed for antibody titers, total IgG and antigen specific IgG and leukocyte populations during the disease progression. This will be useful to study progression of the autoimmune disorders induced which can be correlated with behavior, neurologic and locomotor data.

   Control groups are required in all experiments. We will include 3 groups of animals:
   1. Animals injected with the antigen + CFA; animal model
   2. Animals injected with saline + CFA; control 1, animal without the disease
   3. Animals injected with saline; control 2, animal without any immunogen agent injected

With this approach we will be able to know the specific effect of the antigen, by analyzing the effect of the antigen and the effect of the CFA. It is also important to define the effect of CFA since this has never been tested before and maybe it enhances the pain and discomfort sensation in the animals. In case pain is tested, animals without any intervention have to be included to have the basal/physiological level of this measurement.

The motor, neurologic and behavioral battery set up will depend on the nature of the disorders. Also, from each animal model, the batch of tests will be selected to answer the questions as accurately as possible. Every antigen will induce a specific group of symptoms. Some examples of which we expect to study with the associated symptoms are depicted in the table below.

Table 1. [redacted]

[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

[redacted] before. All possible tests have been mentioned in the Table 2 (below). The combination of the tests selected IS disease specific AND is defined according to the pathology and the research question. The choices of the test that we will perform will be based on the latest literature for each pathology reported (Table 1) and on the availability of the tests in our department. It is important to mention that not all the animals in the same experiment will follow the same set of tests, since some of them are incompatible. Some tests have to be performed regularly as described in the Table 2 and others just once, where some others are terminal experiments.

Table 2. Procedures required characterizing neuropsychiatric animal models. Tests are classified according to the main outcome and the character of it: behavior, neurologic and locomotion. A brief description per test and the degree of invasiveness and discomfort has been included.

Procedure/test	**Description**	**Main outcome**	**Example**	**Invasive/ non invasive// discomfort**	**Behavior/ neurologic/ locomotion/ operational**
1. Alternating Y-maze (Hughes RN., 2004)	The animal is introduced in the center of a Y-shaped maze with three white opaque arms. The number of arm entries and the percent of alternations are calculated.	Willingness to explore new environments. Cognitive working memory.	Individuals with cognitive deficits perform less alternation compared to wild type (WT) animals due to the deficit in cognitive working memory.	Non invasive // Mild discomfort (anxiety)	Behavior (cognition)
2. Black and white	The animal is introduced	Anxiety and	The preference by	Non invasive	Behavior (affect)

test box (Costall B. et al., 1989)	into a box with bright and dark areas. The preference light/dark areas are measured.	exploratory behavior.	dark areas can be silenced by anxiolytic drugs.	// Mild discomfort (anxiety)	
3. Catwalk (Deacon R.M.J., 2013)	The animal traverses a glass plate voluntarily and the footprints are recorded.	Locomotor activity and nociception.	Minimal physical interference and pain behaviors can be detected and analyzed (different patterns) by this method. Analgesic efficiency can be evaluated.	Non invasive // Mild discomfort	Locomotion
4. Cued and Contextual Fear Conditioning (Curzon P. et al., 2009)	The animal is placed in a box where different expected/unexpected stimulus (light, tone, odor) will be presented. The animal will freeze when an unexpected stimulus is presented. The percentage spent freezing and time for extinction will be measured.	Associative learning and memory (amygdala-hippocampus dependent).	Rodent models with cognitive deficits show less freezing during both cued and context freezing after having been conditioned. Furthermore, they show faster extinction of conditioning compared to WT animals.	Non invasive // Moderate discomfort	Behavior (cognition)
5. Electromyography (EMG) (Osuchowski M.F., et al., 2009)	Different electrodes are located in the paw and in the tail. The time required to transport the electric signal is recorded.	Skeletal muscle electric activity.	In MG rats, EMG is useful to record the degeneration of the muscles and the progression disease.	Invasive// Mild discomfort (under anesthesia, terminal experiment)	Neurologic
6. Elevated zero maze test (Kulkarni SK. et al., 2007)	Animals are displaced in an annular or "X" shape elevated platform with open and enclosed quadrants. The time spent in enclosed areas is measured.	Anxiety.	In rodent with cognitive deficits, spent less time in the enclosed arms compared to WT animals, indicating memory deficits.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
7. Encephalography (EEG) (Xie C. et al., 2011)	Brain spontaneous activity is measured and recorded from multiple electrodes placed on the scalp. A video recording is necessary to confirm epileptic seizures.	Electrical brain activity and epileptic seizures.	Epileptic events and encephalopathies can be diagnosed by this method in animal models.	Invasive // Mild discomfort (under anesthesia)	Neurologic
8. Forced swimming tests (Can A. et al., 2012)	The animal is placed in a cylindrical tank with water (the bottom is not reachable and they can not scape from the container). The time spent trying to escape/swim/float is recorded.	Anhedonia, depressive-like behavior	Anti-depressant therapies can be tested using this technique.	Non invasive // Moderate discomfort (anxiety and stress)	Behavior (affect)
9. Horizontal and	The animal is placed in	Motor function,	The animal get a	Non invasive	Locomotion and

fixed bars (Deacon RMJ. 2013)	the middle of a horizontal suspended bar (with different diameters) where they need to walk without falling. In the fixed bars, the different diameters bars are fixed and they need to go from one end of the stick to the base.	orientation and coordination	composite score: time spent on the bar without falling, capacity to reach the base of the apparatus and the diameter of the bar. Animals with neurological or locomotor issues will have difficulties and fall soon.	// Mild discomfort	neurologic
10. Intubation ([redacted] et al., 2015)	Artificial ventilation is provided to the animal through a trachea incision, placing a tube connecting to air flow.	Ensure breading	Electromyography is a procedure where curare is injected, blocking all muscles making artificial ventilation necessary.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Operational
11. Morris Water Maze (Vorhees CV. and Williams MT., 2008)	The rodent is placed in a swimming arena where a submerged platform is located in a strategic place. The time spent to learn will determine the spatial learning and later on the time spent to find the platform will be correlated with the reference memory (the arena is divided by 4 quadrants).	Spatial learning and memory hippocampus dependent. Swimming skills	Rodents with cognitive deficits will spend less time in the target quadrant, because they forgot where it was. The swimming speed can also be measured to determine the locomotion status.	Non invasive // Mild discomfort	Behavior (cognition) and locomotion
12. Nerve conductance speed (NCS) (Osuchowski M. et al., 2009; [redacted] et al., 2015)	Electrodes are placed in the leg and on the tail of the animal. The time required to receive the stimulus from the emitting and the receiving is recorded and correlated with the nervous and muscle status	Nerve damage and neuropathic measurement	Nerve damage can be measured using this technique for example for sciatic nerve impairment. Neuropathy can be tested also as a drug side effect using this technique	Invasive // Mild discomfort (under anesthesia)	Neurologic
13. Object recognition task (Leger M. et al., 2013)	Two similar objects are presented to the animal for the first time and the second time one of them is replaced by a new object. Object exploration duration and habituation times can be analyzed.	Cognition and recognition memory	Animals with cognition impairment will spend more time to recognize the constant and the new object.	Non invasive // Mild discomfort	Behavior (cognition)
14. Olfactory function /Smell threshold concentration test (Yang M. et al.,	Animals are exposed to increasing concentrations of menthol diluted in mineral oil and the minimal menthol	Olfactory function and depression like-behavior	A depressive like behavior is related to decreased olfactory function, so animals with depression will	Non invasive // Mild discomfort	Behavior (affect)

2009; Katzay A. et al., 2014)	concentration that the mouse responds to is recorded. A mouse response is considered positive when it displays a sharp aversive movement away from the source of smell.		need a stronger olfactory stimulus for positive reaction		
15. Open Field Test (Bailey KR. And Crawley JN., 2009)	The animal is placed in a square chamber, divided in areas. A recording system will determine the preference of the animal between the periphery and the central areas. In parallel, distance and speed, rare behaviors, grooming, etc. can be evaluated.	Locomotion, emotional and anxiety.	Rodent models with cognitive deficits or treated with anxiolytic drugs will show reduced time spent in corners due to reduced anxiety.	Non invasive // Mild discomfort (anxiety)	Behavior (affect) and locomotion
16. Parallel rod floor test (Kamens HM and Crabbe JC., 2007)	The animal is placed in a parallel stainless steel rod with a base plate behind. The animal paws are recoded and the number of times they slip through the parallel metal rods and touches the base plate.	Motor coordination (ataxia) and activity.	In rodent with cognitive deficits, locomotion deficits have been observed.	Non invasive //Mild discomfort	Locomotion
17. Perfusion ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is injected in the central venous system with a reagent (PBS or a fixative)/ The liquid is injected in the left atrium and a cut in the right ventricle drain the liquid.	Remove blood and/or preserve the tissue and organs.	Muscles in MG animal models have to be analyzed ab for this, fixed material is necessary because it help to maintain the integrity of the proteins. Brain is another important organ where sometimes the blood has to be removed or the tissue has to be fixed.	Invasive// Mild discomfort (under anesthesia, terminal experiment)	Operational
18. Pre-pulse inhibition (PPI) (Powel SB et al., 2009)	The animal receives a weak prestimulus o prepulse that results in the inhibition of the reaction of the animal after a subsequent strong startling stimulus or pulse (usually acoustic or light stimulus).	Anhedonia, depressive-like behavior	PPI has been used primarily in pharmacological animal models to screen putative antipsychotic medications. It is considered to be one of the most promising neurophysiological indexes for translational research in psychiatry (Hidetoshi T et al.,	Non invasive // Mild discomfort	Behaviour (affect)

			2011).		
19. Rack grabbing test ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and a 300g metal rack has to be grabbed repeatedly (5 times with a resting interval of 30s aprox.).	Strength, weakness and fatigability. Depressive-like behaviors.	MG animal models or other muscle impaired models can be graded using this method by the number of repetitions and the time they grab the rack.	Non invasive //Mild discomfort (anxiety)	Locomotion, Behavior (affect)
20. Resident-intruder tests (Koolhaas JM. et al., 2013)	The animal is placed in a box, first with a sterile female and later with a male from a non-aggressive strain. Social interaction and stereotypical aggressive behaviors are recorded and later analyzed by the frequency of these activities.	Social interaction, stress and aggressiveness/violence.	The social and communication skills and the locomotion can be measured in transgenic mice, where the animals presented more violence. Also the drug effect can be studied in terms of clarity and aggressiveness.	Non invasive //Mild discomfort (anxiety)	Behavior (affect) and locomotion
21. Rotarod (Deacon RMJ. 2013)	The animal is placed in a horizontal cylinder (delimitated on the sides). The cylinder rotate at different increasing speeds and the animal have to walk without falling.	Motor function and coordination	The animal gets a composite score: ledge test, hind limb clasping, gait, kyphosis (locomotion) and time spent. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort (anxiety)	Locomotion and neurologic
22. Social interaction test (Kaidanovich-Bejilin O. et al., 2011; Sato A. et al., 2013)	The animal is placed in a 3 chamber cage. After habituation, the preference between an empty/known animal/unknown animal is measured. The activity is recorded and number and time spend in each department counted.	Natural sociability and preference novel sociability.	Many neuropsychiatric disorders are characterized by social behavior and recognition disruption. Other locomotion and cognitive parameters can be recorded.	Non invasive //Mild discomfort	Behavior and locomotion (affect)
23. Spatial Y-maze, X-maze and V-maze spontaneous alternation test (Lainiola M. et al., 2014)	The animal is placed in the connection of a 3 arm maze and the exploring capacity is measured by the number of arm entries/alternation. Different objects can be placed at the end of the arms.	Cognitive deficits, memory, anxiety	Transgenic animals or drug effect can be analyzed in the cognitive level using this method, measuring the willingness of rodents to explore new environments. Memory deficits can be measured also by the time spent to recognize the objects.	Non invasive //Mild discomfort (anxiety)	Behavior (affect/ cognition)
24. Sucrose	The rodent is free to	Anhedonia and	In chronic stress	Non invasive	Behavior (affect)

preference/anhedonia test (Strekalova T. et al., 2004)	choose between a water/sucrose solutions. The % of preference sucrose/liquid consumed is used as criteria (threshold 65%).	depression-like behaviors	animal model, depression like behavior shows the acuteness of the model with a reduction of sucrose consumption.	// Mild discomfort	
25. Tail suspension test [redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and they need to climb on the top of the hand repeatedly (5 times with a resting interval of 30s approx.).	Strength, weakness, fatigability. Stress and depressive-like behaviors.	In MG rats, this is a parameter used to observe the acuteness of the disease. Animals exposed to tail suspension for longer periods daily develop chronic stress.	Non invasive //Mild discomfort (anxiety)	Locomotion and behavior (affect)
26. Venipuncture	A needle is used to puncture an occluded vessel (saphenous vein or submandibular venous sinus). The blood start dripping and after collecting the required amount, you have to press the area to stop the bleeding.	Obtaining a blood sample.	Blood contains a lot of parameters that can be studied in order to monitor autoimmune state in animal disease models like MG, encephalitis, etc. Drug side effects can also be assessed by blood analysis.	Invasive // Mild discomfort	Operational
27. Von Frey and Hargraves (Krzyzanowska A. And Avedaño C., 2012)	The animal is subjected to different thermic and mechanic increasing intensity stimulus. The maximum intensity the animal can withstand without reaction is recorded as a threshold.	Pain susceptibility/ nociception.	Animal models can be tested by this method to assess the susceptibility to pain which reflect their own welfare. Could be an indirect measurement of therapy efficiency or to detect side effects. It also helps to determine the general health.	Non invasive // Moderate discomfort	Behavior (pain)
28. Weight measurement	Animals are placed in a balance and the weight is recorded.	General health.	Weight is a general measurement related with general health, disease progression (for example in MG) and drug side effects.	Non invasive //Mild discomfort	Operational

All animals will undergo A behavioral-neurological-locomotor battery adjusted to the induced disease, varying from 2-15 the amount of tests/procedures per animal distributed along the experiment ([redacted], et al., 2011; Planaguma J. et al., 2015).

An example of CNS passive transfer models with cognitive impairment, is the one performed by Planaguma J. and colleagues, where the animals are characterized using the novel object recognition in open field and V-maze task, sucrose preference/anhedonia test, tail suspension and forced swimming tests, black and white and elevated plus maze tests, resident-intruder test and horizontal and vertical activity assessment. The tasks are performed once to weekly frequency. In our case, we have designed a set of tasks based on 5 domains (specified in Table 2), since the literature is quite terse and the models we will develop might not follow the same pathological mechanisms having different impairments. Based on these domains, we have select a first line tests which will be the first ones applied to characterize the

animal model:

1) Affect: Elevated zero maze test, sucrose preference test and pre-pulse inhibition
2) Cognition: Alternating Y-maze and object recognition task
3) Motor: Open field and rotarod
4) Pain: Von Frey
5) Neurologic: Electroencephalography

After the analysis of the first results, a more specific batch of test will be applied to the specific animal model, focusing on the domains affected. We expect differences in 2-3 domains and we will undertake a maximum of 3 tasks per domain to characterize the animal model in a proper way in those areas where the phenotype is altered (see Table 2). An example of it is the Resident-intruder test or the social interaction, which will introduce more power to an aggressive or abnormal behavior, already observed when the animal is placed in a box with others. The tests will be chosen based on the non-overlapping of outcomes and the non-alteration (bias) of the results because of the application of all of them together. In case those interactions between tests are observed at mid-term evaluation of the results, each group will be subdivided and the animals will follow different set of tasks, reducing in this way the bias.

For the PNS a different battery of task will be applied based on previous experience or recent literature. An example of the batch of tests performed in an active immunization MG model for a drug experiment is ([redacted] et al., 2010):

1. Motor: Rack grabbing test and tail suspension test
2. Pain: Von Frey and Hargreaves tests
3. Neurologic: nerve conductance speed and electromyography

Besides the cognition, neurologic and locomotor assessment, some other operational task may be applied to the animal models like general observation (not consider a test/task, since the animals will be checked routinely), venipuncture and weight measurements during the experiment and other terminal tasks like intubation or perfusion at the end of the experiment, depending on the specific purpose of the study.

All the tests/procedures applied to the animals are well established procedures under a standard protocol (Standardized operational protocol; SOP) which will reduce the bias of the experiment, since in these cases, interaction between the different tasks are already described and we will not make these specific set of tests for the experimental animals. In case it is necessary to include tasks that will affect each other because of the research question, the groups will be divided in subgroups which will follow different sets of tests. On the other hand, another measure to reduce the bias is the design of the tests performed in each experiment, which will be defined by the literature available currently before the performance of the experiments and also based on the expertise of people who has large experience in the behavior test. Under their advice, the tests will be chosen following a stress increasing scale throughout the experiment (in example, an open field will be always performed before a forced swimming test). It is important to mention that all observers (minimum of 2 independent researchers) will be blinded to different groups when performing the tasks.

For this appendix the go/ no-go criteria are the following:

1. Animal phenotype
    a) Robust and standard: analysis on the different behavior, neurologic and/or locomotion tasks will be performed as well as *in vitro* analysis defining the levels of different markers involved in autoimmunity like autoantibodies (specific and unspecific), inflammation cells and molecules e.g. plasma cells and cytokines. For standard animal models, the non-reproduction of the symptomatology and other markers is a direct no-go criteria, taking into account the variance between experiments [redacted] et al., 2015). For novel animal models, if no differences equal or higher than 10% between the disease and the control group are found, a no-go decision will be followed. In this case, the experimental conditions of the model will be studied in detail, and changes (e.g. species, gender, route of administration, frequency, dose, adjuvants, etc.) will be implemented to try to generate a robust and standard animal model. If no differences are found after modifying the strategy, we will report the results as a non-successful or non-viable model for the specific antigen of interest.
    b) Severe: the symptomatology of the animal model will be observed and in case of severe discomfort and/or pain because of the intervention procedures (e.g. immunization), analgesic

will be applied and if even using this the animal keep suffering, a no-go decision will be followed as explained in point a). If animals reach humane endpoints (section J), they will be sacrificed.

2. Test battery

After the analysis of the results of the battery tests, a go/no go decision will followed. In case an animal model shows differences in one of the domains and not in the other, the number of tasks assessing this parameter will be increased and the other test removed from the batch. Moreover, some tests included in Table 2 will be performed in case a strange behavior is observed during routine observation. It is important to mention that, the behavioral battery may change based on results generated from the first groups tested (based on a go/no-go decision after a first characterization in case of CNS active immunization models). For example:

a) When the animal model is new, a characterization of the results in each test will provide an overview of the effect of each antigen specific immunization in the animal model, so a go decision will be followed in this case up to enough reliable data is available.
b) In case of new animal models when a test has been shown (see previous point a)) not to detect differences between groups using a positive and a negative control, it will be discarded from the behavioral battery for this specific animal model (this indicates that the test for that particular model is not useful (no go decision)).
c) When the animal model is well characterized and the results from the test are unexpected, showing no differences between the groups, a no-go decision will be followed.

For the postmortem study, CSF and blood will be collected for analysis of different molecule levels (e.g. total immunoglobulin levels and specific isotypes and antigen specific immunoglobulin or other factors affected) and cell populations will be studied as well to see how different immune system related cells and molecules can vary due to the disease. On the other hand, tissues will be collected to be analyzed. Immune related organs – thymus, spleen, lymph nodes, bone marrow –, brain, muscles and other organs which can be affected specifically by the disease developed – intestines, gut, sciatic nerve, liver, kidney, etc.-. For tissue collection, based on the research question to be answered, different treatments can be applied to the tissue, being necessary to include in some experiments perfusion with different reagents (glutaraldehyde, paraformaldehyde, etc.) of the animals to obtain fixed tissue (e.g. for MG, 2% glutaraldehyde perfused muscles are necessary to study the neuromuscular junction structure).

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

The numbers of animals will be kept to a minimum by collaboration with people who have extensive experience in the generation of autoimmune animal models.
The research strategy will be based on the use of the most informative and descriptive test set for each disease model under the advice of experts in the field, own experience and the use of the literature. We will try to include as many tests as possible taking always into account the bias error and also we will avoid superfluous repetitions. As mentioned above, go/no-go decisions will be taken according to the pathology. If no differences are found using a test already reported to show significant differences, the experiment will be halted. In case there is no previous report about the test, the experiment will continue as a characterization (see above paragraph test battery).

We will use the effect size as a quantitative measurement of the strength of the phenotype of the animal model. Taking into account the next assumptions: a very large-huge effect size (d=1.20-1.40; Sawilowsky, S. Journal of Modern Applied Statistical Methods, 2009) and a power of 0.80 (Cohen J., Statistical Power Analysis for the Behavioral Sciences, 1988), with an α=0.05 for two-tiled and α=0.025 for one tailed, we will need 9-12 animals per group.

As parameters we will use, in active immunization animal models: IgG titers and weight measurements with some phenotype characteristics (in case of CNS, all the tests proposed in the 5 domains that we will perform in the first pilot) to see whether there are differences or not between the animal model and the controls.

B. The animals

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

For this experiment, rodents, including mice and rats will be used. Different species, strain, and genetic background (also WT) animals can be included. The species (3), strain based on the genetic background, gender (2) and age range will be adjusted according to the literature to the most suitable. According to the induced nervous system autoimmune pathology, the onset severity and progression of the disease can vary and also the extension of the model.

- NMJ animal model using AChR is the only model already well established. We will use Lewis female rat, 8-12 weeks of age, are the most susceptible strain and gender for AChR active immunization [redacted] et al., 2015). Another point in favor to females is that the size of the animal is also important since the amounts of antigen required and the doses of drugs are directly correlated with the weight of them. Other antigens presents in the NMJ can be used to generate the animal models. In these cases, we will use at first the study design used for AChR animal model and the recent literature on the topic (currently there is no information available since we did not define the novel antigen yet).
- For other active immunization models, we will follow the literature available (Denic A. et al., 2013) and in case there is none, we will follow the criteria of other CNS autoimmune models (Planaguma J. et al., 2015, Jones M.V. et al., 2012) or other PNS active immunization models like MG ([redacted] et al., 2015). The prevalence of the disorder gender specific in the human population can also be taken into account since the last aim of all research proposes are to reach the human level.

For the animal models of active immunization we will generate a maximum of 5 animal models. For these studies the amount needed depends on the purpose of the animal model (behavior, neurological and/or locomotor description, mechanisms of pathogenicity studies, drug test, new therapies implementation, etc.). We expect a max. of 204 individuals (in example, taking into account 4 groups; disease (20 animal) /healthy (12 animal) * condition "X" /control, in triplicate experiments: ((20*2+12*2)*3=192) an average of the animal number included in each active immunization models, since the genetic background of the animals, the number of conditions to be tested, etc. can be modified (for example, in case pain tests will be included, a non-immunized control group has to be included, so 12 more animals have to be included or the necessity to divide the groups in 2 subgroups because of the tasks overlapping: for example when a task measuring anxiety and another one measuring cognitive impairment cannot be applied in the same animal because one will interfere with the other and the results will not be reliable). In this calculation we have already taken into account that some animals will have the same conditions but will be sacrificed in different way according to the tissue analysis requirement and the high variability existent in active immunization models. In total we will not use more than 1020 animals for active immunization models (204*5=1020 animals).

Summarizing, a total of 1020 animals (816 rats and 204 mice) will be included in the "Active immunization in rodents" appendix.

All animals will be obtained from a registered supplier or a researcher who have specific animal models in case the registered supplier doesn't have it.

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.

Animal models of autoimmune diseases are required to study the *in vivo* pathogenic mechanisms, which will help to understand the procedures underlying the disease. There are no other *in vitro* experimental approaches that can give us this information, because the disease development in this case is very complex and dependent on the interplay of immune system and nervous system. Additionally, we aim to prove that the autoimmune disease can induce neurological and psychiatric symptoms which are difficult to measure in lower organisms.
The number of animals will be keep to the minimum that can give statistical differences by doing power calculations for sample size and implementing go/no-go criteria we further limit the amount of animals used.
Refinement will take place by using anaesthesia and analgesia for the invasive procedures, including pain due to the use of adjuvants during the immunization and the discomfort generated by the development of the pathology. Incomplete FA (IFA) will be used in case that repetitive immunizations are required (Li, 2004).

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

We are going to adapt the accommodation and the care to the need of the animals. To limit animal discomfort; anaesthesia and analgesia will be used to reduce the discomfort of the animals during the invasive procedures, like nerve conductance or for the terminal experiments like electromyography using curare where the animals have to be intubated and receive mechanical ventilation (for more details see Table 1). Animals which are going to follow pain susceptibility test could not be under anesthesia (will not have anesthesia during a certain period before the test is performed). Usually, pain tests are run in the middle of the experiment, enough time after the immunization and before the terminal experiments, so the anesthesia is not interfering in any case. Because of this, if some animal present high discomfort, the subject will be withdrawn (in any case, this is not what we expect, since these specific tests are performed in chronic models and the human endpoints are always taken into account).

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

☒ 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.

Animals will be monitored daily. Anaesthetics and analgesics will be used to reduce the pain and discomfort of the animals due to some procedures (immunization, NCS and EMG). In some cases (e.g. when it is important to asses pain as an outcome parameter), pain tests are going to be run during the experiment as a part of the model characterization and can also be used as a welfare measurment (in MG models pain is expected due to the imunization but in CNS models there is no previous data reporting this).

I. Other aspects compromising the welfare of the animals

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

We can expect adverse effect of the immunization and of the induced disease *per se*, for example, EAMG cause weakness and fatigability (including problems with food intake and difficulties to breathe or the appearance of elephant teeth) and in case of CNS, memory and some neurological and locomotors impaired abilities can be expected, which generate stress as explained more in detail in section A.

There are no adverse effects are expected in relation to the venipuncture or the NCS, neither in the behaviour nor in pain susceptibility tests.

Explain why these effects may emerge.

An unwanted effect could be observed because of immune reaction against the antigen and the adjuvant. The animal will undergo inflammation in the area of injection with possible pain. Pain killers will be administered if necessary. Furthermore, systemic inflammation might occur if the antibodies produced by the animals start reacting with endogenous proteins. Many of these antigens have never been used in rodent models and we cannot know how symptoms differ from the human disease onset and progression.

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

Regular monitoring will prevent and minimize the discomfort since the animals are going to receive pain killer, anesthetics and/or analgesics in case they are necessary. The immunization site will be checked the days after the intervention to be sure that no complications appear. Human endpoints will be defined specific for the pathology studied and in case the animal shows a severe discomfort, euthanasia will be performed if the individual reach that status.

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.

Humane endpoints will be applied in case of rapid weight loss (>20% within 3 days without any improvement–some autoimmune models have a transient acute phase of the disease which

spontaneously recovers), severe acute inflammation at the immunization point (untreatable by the definition of the veterinarian). The following endpoints are unlikely for completeness we include them since some phenotypes have never been tested before. These includes prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
In case of generalized unexpected severe adverse reaction the experiment will be halted.

Indicate the likely incidence.

The severity of the induced pathology fluctuates since acute or chronic illness can be induced depending on the dose, frequency and duration of the immunization (Lennon V. et al., 1975; [redacted] et al., 2015).

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').

All the animals will experience some degree of discomfort. The intermediate analysis of the behavioral, neurologic and/or locomotion tests cause mild to moderate discomfort. Operational procedures generate mild discomfort in 100% cases, however, immunization evokes moderate discomfort (single immunization with CFA and repetitive immunizations with IFA). Since all the animals will undergo immunization (except the control group for pain tests, which will not have CFA injection), the added discomfort of all tasks is moderate.

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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.

Animals will be sacrificed at the end of the experiment.
The sacrifice of the animals will be done according to the analysis requirements, in this case, based on the tissue characteristics. If fixed tissues have to be studied, perfusion is necessary to preserve the organ/tissue in an optimal way.

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

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

☒ Yes

Appendix
Description animal procedures

1) This appendix should be enclosed with the project proposal for animal procedures.
2) A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
3) For more information, see our website (www.centralecommissiedierproeven.nl).
4) 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'.	10700
1.2	Provide the name of the licenced establishment.	University Maastricht
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: Passive immunization in rodents

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.

This appendix describes the development of passive immunization rodent models for autoimmune mediated diseases that target antigens in the nervous system.

[redacted]

[redacted]

The primary outcome is the generation of autoimmune models of the nervous system to study the pathogenic mechanisms of the autoimmune disorders affecting the nervous system. The measures will include behavioral (symptomatic), neurological and locomotor tests, followed by post mortem analysis to describe the pathogenic mechanisms of the autoimmune diseases.

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

The immunization varies on the animal model as described above. The frequency is also determined by several parameters like the immunogenicity and stability of the antibodies, the severity of the induced disease, etc. with a minimum of a unique immunization at the beginning to a constant perfusion of the antibody until the end of the experiment. For peripheral injections, i.p. injections are the most common ones unless the antibody target is highly expressed in the peritoneal cavity, being then i.v. administration the optimal. For intrathecal injection, a constant intra-ventricular infusion via a minipump is optimally used. In shorter experiments (<1 week) only one or two intra-ventricular injections would be considered. In case the antibody has to reach the CNS and the injection is peripheral, BBB permeabilization reagents will be used, together with the antibodies, as described above. The immunization site will be checked the days after the intervention to be sure complications will not appear.

Control groups are required in all experiments. We will include a control without the disease (a non-pathogenic antibody is going to be injected instead of the pathogenic antibodies). The control group will be treated equally as the study group, meaning that permeabilize reagents will be also injected to control group if required. In case the study is based on the capacity of the antibody to penetrate the BBB, the study group is composed by 2 groups (antibody with and without the permeabilization), 2 control groups will be required (antibody without permeabilization and permeabilization without antibody).

The motor, neurologic and behavioral battery set up will depend on the nature of the disorders. Also, from each animal model, the batch of tests will be selected to answer the questions as accurately as possible. Every antibody will induce a specific group of symptoms. Some examples of which we expect to study with the associated symptoms are depicted in the table below.

Table 1. [redacted]

Animal procedures selected will depend on the purpose of the study as mentioned before. All possible tests have been mentioned in the Table 2 (below). The combination of the tests selected disease specific is defined according to the pathology and the research question. All the choices about the test that we will perform will be based on the latest literature for each pathology reported (Table 1) and on the availability of the tests in our department. It is important to mention that not all the animals in the same experiment will follow the same set of tests, since some of them are incompatible. Some tests have to be performed regularly as described in the Table Y and others just once, where some others are terminal experiments.

Table 2. Procedures required characterizing neuropsychiatric animal models. Tests are classified according to the main outcome and the character of it: behavior, neurologic and locomotion. A brief description per test and the degree of invasiveness and discomfort has been included.

Procedure/test	**Description**	**Main outcome**	**Example**	**Invasive/ non invasive// discomfort**	**Behavior/ neurologic/ locomotion/ operational**
1. Alternating Y-maze (Hughes RN., 2004)	The animal is introduced in the center of a Y-shaped maze with three white opaque arms. The number of arm entries and the percent of alternations are calculated.	Willingness to explore new environments. Cognitive working memory.	Individuals with cognitive deficits perform less alternation compared to wild type (WT) animals due to the deficit in cognitive working memory.	Non invasive // Mild discomfort (anxiety)	Behavior (cognition)
2. Black and white test box (Costall B. et al., 1989)	The animal is introduced into a box with bright and dark areas. The preference light/dark areas are measured.	Anxiety and exploratory behavior.	The preference by dark areas can be silenced by anxiolytic drugs.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
3. Catwalk (Deacon R.M.J., 2013)	The animal traverses a glass plate voluntarily and the footprints are recorded.	Locomotor activity and nociception.	Minimal physical interference and pain behaviors can be detected and analyzed (different patterns) by this method. Analgesic efficiency can be evaluated.	Non invasive // Mild discomfort	Locomotion

4. Cued and Contextual Fear Conditioning (Curzon P. et al., 2009)	The animal is placed in a box where different expected/unexpected stimulus (light, tone, odor) will be presented. The animal will freeze when an unexpected stimulus is presented. The percentage spent freezing and time for extinction will be measured.	Associative learning and memory (amygdala-hippocampus dependent).	Rodent models with cognitive deficits show less freezing during both cued and context freezing after having been conditioned. Furthermore, they show faster extinction of conditioning compared to WT animals.	Non invasive // Moderate discomfort	Behavior (cognition)
5. Electromyography (EMG) (Osuchowski M.F., et al., 2009)	Different electrodes are located in the paw and in the tail. The time required to transport the electric signal is recorded.	Skeletal muscle electric activity.	In MG rats, EMG is useful to record the degeneration of the muscles and the progression disease.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Neurologic
6. Elevated zero maze test (Kulkarni SK. et al., 2007)	Animals are displaced in an annular or "X" shape elevated platform with open and enclosed quadrants. The time spent in enclosed areas is measured.	Anxiety.	In rodent with cognitive deficits, spent less time in the enclosed arms compared to WT animals, indicating memory deficits.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
7. Encephalography (EEG) (Xie C. et al., 2011)	Brain spontaneous activity is measured and recorded from multiple electrodes placed on the scalp. A video recording is necessary to confirm epileptic seizures.	Electrical brain activity and epileptic seizures.	Epileptic events and encephalopathies can be diagnosed by this method in animal models.	Invasive // Mild discomfort (under anesthesia)	Neurologic
8. Forced swimming tests (Can A. et al., 2012)	The animal is placed in a cylindrical tank with water (the bottom is not reachable and they can not scape from the container). The time spent trying to escape/swim/float is recorded.	Anhedonia, depressive-like behavior	Anti-depressant therapies can be tested using this technique.	Non invasive // Moderate discomfort (anxiety and stress)	Behavior (affect)
9. Horizontal and fixed bars (Deacon RMJ. 2013)	The animal is placed in the middle of a horizontal suspended bar (with different diameters) where they need to walk without falling. In the fixed bars, the different diameters bars are fixed and they need to go from one end of the stick to the base.	Motor function, orientation and coordination	The animal get a composite score: time spent on the bar without falling, capacity to reach the base of the apparatus and the diameter of the bar. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort	Locomotion and neurologic
10. Intubation	Artificial ventilation is	Ensure breading	Electromyography is a	Invasive //	Operational

[redacted]. et al., 2015)	provided to the animal through a trachea incision, placing a tube connecting to air flow.		procedure where curare is injected, blocking all muscles making artificial ventilation necessary.	Mild discomfort (under anesthesia, terminal experiment)	
11. Morris Water Maze (Vorhees CV. and Williams MT., 2008)	The rodent is placed in a swimming arena where a submerged platform is located in a strategic place. The time spent to learn will determine the spatial learning and later on the time spent to find the platform will be correlated with the reference memory (the arena is divided by 4 quadrants).	Spatial learning and memory hippocampus dependent. Swimming skills	Rodents with cognitive deficits will spend less time in the target quadrant, because they forgot where it was. The swimming speed can also be measured to determine the locomotion status.	Non invasive // Mild discomfort	Behavior (cognition) and locomotion
12. Nerve conductance speed (NCS) (Osuchowski M. et al., 2009; [redacted] et al., 2015)	Electrodes are placed in the leg and on the tail of the animal. The time required to receive the stimulus from the emitting and the receiving is recorded and correlated with the nervous and muscle status	Nerve damage and neuropathic measurement	Nerve damage can be measured using this technique for example for sciatic nerve impairment. Neuropathy can be tested also as a drug side effect using this technique	Invasive // Mild discomfort (under anesthesia)	Neurologic
13. Object recognition task (Leger M. et al., 2013)	Two similar objects are presented to the animal for the first time and the second time one of them is replaced by a new object. Object exploration duration and habituation times can be analyzed.	Cognition and recognition memory	Animals with cognition impairment will spend more time to recognize the constant and the new object.	Non invasive // Mild discomfort	Behavior (cognition)
14. Olfactory function /Smell threshold concentration test (Yang M. et al., 2009; Katzay A. et al., 2014)	Animals are exposed to increasing concentrations of menthol diluted in mineral oil and the minimal menthol concentration that the mouse responds to is recorded. A mouse response is considered positive when it displays a sharp aversive movement away from the source of smell.	Olfactory function and depression like-behavior	A depressive like behavior is related to decreased olfactory function, so animals with depression will need a stronger olfactory stimulus for positive reaction	Non invasive // Mild discomfort	Behavior (affect)
15. Open Field Test (Bailey KR. And Crawley JN., 2009)	The animal is placed in a square chamber, divided in areas. A recording system will determine the	Locomotion, emotional and anxiety.	Rodent models with cognitive deficits or treated with anxiolytic drugs will show	Non invasive // Mild discomfort (anxiety)	Behavior (affect) and locomotion

	preference of the animal between the periphery and the central areas. In parallel, distance and speed, rare behaviors, grooming, etc. can be evaluated.		reduced time spent in corners due to reduced anxiety.		
16. Parallel rod floor test (Kamens HM and Crabbe JC., 2007)	The animal is placed in a parallel stainless steel rod with a base plate behind. The animal paws are recoded and the number of times they slip through the parallel metal rods and touches the base plate.	Motor coordination (ataxia) and activity.	In rodent with cognitive deficits, locomotion deficits have been observed.	Non invasive // Mild discomfort	Locomotion
17. Perfusion [redacted] et al., 2010; [redacted] et al., 2015)	The animal is injected in the central venous system with a reagent (PBS or a fixative)/ The liquid is injected in the left atrium and a cut in the right ventricle drain the liquid.	Remove blood and/or preserve the tissue and organs.	Muscles in MG animal models have to be analyzed ab for this, fixed material is necessary because it help to maintain the integrity of the proteins. Brain is another important organ where sometimes the blood has to be removed or the tissue has to be fixed.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Operational
18. Pre-pulse inhibition (PPI) (Powel SB et al., 2009)	The animal receives a weak prestimulus o prepulse that results in the inhibition of the reaction of the animal after a subsequent strong startling stimulus or pulse (usually acoustic or light stimulus).	Anhedonia, depressive-like behavior	PPI has been used primarily in pharmacological animal models to screen putative antipsychotic medications. It is considered to be one of the most promising neurophysiological indexes for translational research in psychiatry (Hidetoshi T et al., 2011).	Non invasive // Mild discomfort	Behaviour (affect)
19. Rack grabbing test ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and a 300g metal rack has to be grabbed repeatedly (5 times with a resting interval of 30s aprox.).	Strength, weakness and fatigability. Depressive-like behaviors.	MG animal models or other muscle impaired models can be graded using this method by the number of repetitions and the time they grab the rack.	Non invasive // Mild discomfort (anxiety)	Locomotion, Behavior (affect)
20. Resident-intruder tests	The animal is placed in a box, first with a sterile	Social interaction, stress and	The social and communication skills	Non invasive // Mild	Behavior (affect) and locomotion

(Koolhaas JM. et al., 2013)	female and later with a male from a non-aggressive strain. Social interaction and stereotypical aggressive behaviors are recorded and later analyzed by the frequency of these activities.	aggressiveness/violence.	and the locomotion can be measured in transgenic mice, where the animals presented more violence. Also the drug effect can be studied in terms of clarity and aggressiveness.	discomfort (anxiety)	
21. Rotarod (Deacon RMJ. 2013)	The animal is placed in a horizontal cylinder (delimitated on the sides). The cylinder rotate at different increasing speeds and the animal have to walk without falling.	Motor function and coordination	The animal get a composite score: ledge test, hind limb clasping, gait, kyphosis (locomotion) and time spent. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort (anxiety)	Locomotion and neurologic
22. Social interaction test (Kaidanovich-Bejilin O. et al., 2011; Sato A. et al., 2013)	The animal is placed in a 3 chamber cage. After habituation, the preference between an empty/known animal/unknown animal is measured. The activity is recorded and number and time spend in each department counted.	Natural sociability and preference novel sociability.	Many neuropsychiatric disorders are characterized by social behavior and recognition disruption. Other locomotion and cognitive parameters can be recorded.	Non invasive // Mild discomfort	Behavior and locomotion (affect)
23. Spatial Y-maze, X-maze and V-maze spontaneous alternation test (Lainiola M. et al., 2014)	The animal is placed in the connection of a 3 arm maze and the exploring capacity is measured by the number of arm entries/alternation. Different objects can be placed at the end of the arms.	Cognitive deficits, memory, anxiety	Transgenic animals or drug effect can be analyzed in the cognitive level using this method, measuring the willingness of rodents to explore new environments. Memory deficits can be measured also by the time spent to recognize the objects.	Non invasive // Mild discomfort (anxiety)	Behavior (affect/ cognition)
24. Sucrose preference/anhedonia test (Strekalova T. et al., 2004)	The rodent is free to choose between a water/sucrose solutions. The % of preference sucrose/liquid consumed is used as criteria (threshold 65%).	Anhedonia and depression-like behaviors	In chronic stress animal model, depression like behavior shows the acuteness of the model with a reduction of sucrose consumtion.	Non invasive // Mild discomfort	Behavior (affect)
25. Tail suspension test ([redacted]. et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and they need to climb on the top of the hand repeatedly (5 times	Strength, weakness, fatigability. Stress and depressive-	In MG rats, this is a parameter used to observe the acuteness of the disease.	Non invasive // Mild discomfort (anxiety)	Locomotion and behavior (affect)

	with a resting interval of 30s approx.).	like behaviors.	Animals exposed to tail suspension for longer periods daily develop chronic stress.		
26. Venipuncture	A needle is used to puncture an occluded vessel (saphenous vein or submandibular venous sinus). The blood start dripping and after collecting the required amount, you have to press the area to stop the bleeding.	Obtaining a blood sample.	Blood contains a lot of parameters that can be studied in order to monitor autoimmune state in animal disease models like MG, encephalitis, etc. Drug side effects can also be assessed by blood analysis.	Invasive // Mild discomfort	Operational
27. Von Frey and Hargraves (Krzyzanowska A. And Avedaño C., 2012)	The animal is subjected to different thermic and mechanic increasing intensity stimulus. The maximum intensity the animal can withstand without reaction is recorded as a threshold.	Pain susceptibility/ nociception.	Animal models can be tested by this method to assess the susceptibility to pain which reflect their own welfare. Could be an indirect measurement of therapy efficiency or to detect side effects. It also helps to determine the general health.	Non invasive // Moderate discomfort	Behavior (pain)
28. Weight measurement	Animals are placed in a balance and the weight is recorded.	General health.	Weight is a general measurement related with general health, disease progression (for example in MG) and drug side effects.	Non invasive // Mild discomfort	Operational

[redacted]

[redacted]

Besides the cognition, neurologic and locomotor assessment, some other operational task may be applied to the animal models like general observation (not consider a test/task, since the animals will be checked routinely), venipuncture and weight measurements during the experiment and other terminal tasks like intubation or perfusion at the end of the experiment, depending on the specific purpose of the study.

The experiments will be run for shorter periods than active immunization studies (Appendix 2). For example, the passive immunization in MG is terminated 48-72h after induction (Kusner LL, et al., 2015). For other antigens from the CNS such as for NMDAr encephalitis, animals will be sacrificed 5-46 days after immunization (Planaguma J. et al., 2014) or a neuromyelitis optica (NMO) animal model which is injected with antibodies anti-aquaporin-4, which it takes 14 days (Kinoshita M. et al., 2009). Longer experiments studying the chronic pathological role of the antibody will reach maximum 46 days [redacted]. et al., 2016 under revision).

All the tests/procedures applied to the animals are well established procedures under a standard protocol (Standardized operational protocol; SOP) which will reduce the bias of the experiment, since in these cases, interaction between the different tasks are already described and we will not make these specific set of tests for the experimental animals. In case it is necessary to include tasks that will affect each other because of the research question, the groups will be divided in subgroups which will follow different sets of tests. On the other hand, another measure to reduce the bias is the design of the tests performed in each experiment, which will be defined by the literature available currently before the performance of the experiments and also based on the expertise of people who has large experience in the behavior test. Under their advice, the tests will be chosen following a stress increasing scale throughout the experiment (in example, an open field will be always performed before a forced swimming test). It is important to mention that all observers (minimum of 2 independent researchers) will be blinded to different groups when performing the tasks.

For this appendix the go/ no-go criteria are the following:

1. Animal phenotype
    a) Robust and standard: analysis on the different behavior, neurologic and/or locomotion tasks will be performed as well as *in vitro* analysis defining the levels of different markers involved in autoimmunity like autoantibodies (specific and unspecific), inflammation cells and molecules e.g. plasma cells and cytokines. For standard animal models, the non-reproduction of the symptomatology and other markers is a direct no-go criteria, taking into account the variance between experiments. For novel animal models, if no differences equal or higher than 10% between the disease and the control group are found, a no-go decision will be followed. In this case, the experimental conditions of the model will be studied in detail and changes (e.g. species, gender, route of administration, frequency, dose, adjuvants, etc.) will be implemented to try to generate a robust and standard animal model. If no differences are found after modifying the strategy, we will report the results as a non-successful OR NON-VIABLE model for the specific antigen of interest.
    b) Severe: the symptomatology of the animal model will be observed and in case of severe discomfort and/or pain, because of the intervention procedures (e.g. immunization), analgesic will be applied and if even using this the animal keep suffering, a no-go decision will be followed and as explained in point a), some changes will be implemented.

2. Test battery
   After the analysis of the results of the battery tests, a go/no go decision will followed. In case an animal model shows differences in one of the domains and not in the other, the number of tasks assessing this parameter will be increased and the other test removed from the batch. Moreover, some tests included in Table 2 will be performed in case a strange behavior is observed during routine observation. It is important to mention that, the behavioral battery may change based on results generated from the first groups tested (based on a go/no-go decision after a first characterization in case of CNS active immunization models). For example:
   a) When the animal model is new, a characterization of the results in each test will provide an overview of the effect of each antigen specific immunization in the animal model, so a go decision will be followed in this case up to enough reliable data is available.
   b) In case of new animal models when a test has been shown (see previous point a)) not to detect differences between groups using a positive and a negative control, it will be discarded from the behavioral battery for this specific animal model (this indicates that the test for that particular model is not useful (no go decision)).
   c) When the animal model is well characterized and the results from the test are unexpected, showing no differences between the groups, a no-go decision will be followed.

For the postmortem study, CSF and blood will be collected for analysis of different molecule levels (e.g. total immunoglobulin levels and specific isotypes and antigen specific immunoglobulin or other factors affected) and cell populations will be studied as well to see how different immune system related cells and molecules can vary due to the disease. On the other hand, tissues will be collected to be analyzed. Immune related organs – thymus, spleen, lymph nodes, bone marrow –, brain, muscles and other organs which can be affected specifically by the disease developed – intestines, gut, sciatic nerve, liver, kidney, etc.-. For tissue collection, based on the research question to be answered, different treatments can be applied to the tissue, being necessary to include in some experiments perfusion with different reagents (glutaraldehyde, paraformaldehyde, etc.) of the animals to obtain fixed tissue (e.g. for MG, 2% glutaraldehyde perfused muscles are necessary to study the neuromuscular junction structure).
The techniques we are going to use during this study are: cell based assay (CBA), immunohistochemistry (IHQ), immunofluorescent (IF), radioimmunoassay (RIA), ELISA, Western blot/Dot blot, Flow cytometry, polymerase chain reaction (PCR), electron microscopy can be used and are already operational in our group, based on the literature.

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

The numbers of animals will be kept to a minimum by collaboration with people who have extensive experience in the generation of autoimmune animal models.
The research strategy will be based on the use of the most informative and descriptive test set for each disease model under the advice of experts in the field, own experience and the use of the literature. We will try to include as many tests as possible taking always into account the bias error and also we will avoid superfluous repetitions. As mentioned above, go/no-go decisions will be taken according to the pathology. If no differences are found using a test already reported to show significant differences, the experiment will be halted. In case there is no previous report about the test, the experiment will continue as a characterization (see above paragraph test battery).

We will use the effect size as a quantitative measurement of the strength of the phenotype of the animal model. Taking into account the next assumptions: a very large-huge effect size (d=1.20-1.40; Sawilowsky, S. Journal of Modern Applied Statistical Methods, 2009) and a power of 0.80 (Cohen J., Statistical Power Analysis for the Behavioral Sciences, 1988), with an α=0.05 for two-tiled and α=0.025 for one tailed, we will need 9-12 animals per group.

As parameters we will use, in active immunization animal models: IgG titers and weight measurements with some phenotype characteristics (in case of CNS, all the tests proposed in the 5 domains that we will perform in the first pilot) to see whether there are differences or not between the animal model and the controls.
In case of passive transfer animal models, weight measurements and the outcome of the tests performed

to cover the 5 domains will be taking into account.

B. The animals

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

For this experiment, rodents, including mice and rats will be used. Different species, strain, and genetic background (also WT) animals can be included. The specie (3), strain based on the genetic background, gender (2) and age range will be adjusted according to the literature to the most suitable. According to the induced nervous system autoimmune pathology, the onset severity and progression of the disease can vary and also the extension of the model.

It is intended to use wild type animals (with or without LPS/encephalitogenic T-cells injection to permeabilize the BBB), models with and without an impaired BBB (i.e. ApoE$^{-/-}$) for brain targets and human transgenic models (i.e. epsilon or gamma AChR subunits (AChRε, AChRγ)) for the protein of interest to mimic the human pathology.
Based on previous literature for NDMAr encephalitis model; wild type C57Bl/6 can be used to mimic memory deficits, and anhedonic and depressive-like behaviors but not locomoter or epileptic phenotypes (Planaguma L. et al., 2014).
We have already experience with the MG model using female Lewis rats aged 10-12 weeks, which is suitable to distinguish the clinical characteristics. C57Bl/6J mice are recommended to study regulatory functions (Kusner LL. et al., 2015).

In general, we will use adult animals for experiments. However, it is known that many factors from the mother can affect the fetus. Antibodies are an example of these molecules that can cross the placental membrane and affect the fetus, even when the mother does not have symptoms, since the antibodies can target only fetal antigens, such as AChRγ. This has been investigated in autism spectrum disorder, where the mothers from autistic children showed a specific band against fetal brain but not against adult brain, meaning that the autoantibodies transferred by the mothers can recognize the fetal proteins, affecting the child. However, this bands were not found in those mothers with normal children, showing the specificity (Braunschweig D. et al., 2008). This can be also the case of other neuropsychiatric disorders like schizophrenia or bipolar disorder, among others. Because of this, we would like to include pregnant animals to study placenta transfer of antibodies and effects of those antibodies on the offspring viability and on the fetus/neonate.

For the animal models of passive immunization we will generate a maximum of 8 animal models (defined by the time period of 5 years). An estimated distribution can be: 2 animal studies for MG (PTMG), 5 CNS autoantibody studies and 1 model generated with autoantibodies generated *in vivo* (Appendix 1) but since the source of the autoantibodies is mainly from patients, is completely dependent on this. For these studies we will need about 30 animals per group (passive transfer autoimmune models are more robust since autoantibodies are directly administrated to the animal) and the same amounts of control animals.
When studying the role of the BBB, there will be 4 study groups consisting of:

1. Disrupted BBB – pathogenic immunoglobulin
2. Disrupted BBB – nonpathogenic immunoglobulin
3. Non disrupted BBB (wild type) – pathogenic immunoglobulin
4. Non disrupted BBB (wild type) – nonpathogenic immunoglobulin

For the development of all animal models, using a maximum of 8 antigens, this will be a total maximum of 960 animals (30*4*8=960). Likely, we will be able to reduce these numbers by testing different autoantibody targets at the same time. For example, we expect to study AMPAr, GABABr and NMDAr (common outcome parameters) which could all be injected in parallel and thereby we would only need one control group for the 3 antigens. Also, some control group can be saved in case we want to compare the incidence of the BBB disrupted models in the pathology, since the non disrupted groups can be shared in this case.
In this calculation we have already taken into account that some animals will have the same conditions but will be sacrificed in different way according to the tissue analysis requirement and the high variability existent in passive immunization models.

Summarizing, a total of 960 animals (600 rats and 360 mice) will be included in the "Passive immunization in rodents" appendix.

All animals will be obtained from a registered supplier or a researcher who have specific animal models in case the registered supplier doesn't have it.

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.

Animal models of autoimmune diseases are required to study the *in vivo* pathogenic mechanisms, which will help to understand the procedures underlying the disease. Autoantibodies from a specific patient can be individually studied, increasing the personification of the pathogenic tracks in each patient and prove that the autoantibodies are the cause of the pathology, which is difficult to measure in lower organisms. Increasing the knowledge in this area will help the clinicians to apply more efficient treatments to the patients and to test these drugs, also *in vivo* experiments using the disease model are required. There are no other *in vitro* experimental approaches that can give us this information.
The number of animals will be keep to the minimum that can give statistical differences by combining different autoantibodies with similar outcomes and sharing the control groups as explained above. By doing power calculations for sample size and implementing go/no-go criteria we further limit the amount of animals used.
Refinement will take place by using anaesthesia and analgesia for the invasive procedures.

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

We are going to adapt the accommodation and the care to the need of the animals. To limit animal discomfort; anaesthesia and analgesia will be used to reduce the discomfort of the animals during the invasive procedures, like nerve conductance or for the terminal experiments like electromyography using curare where the animals have to be intubated (for more details see Table 1). Animals which are going to follow pain susceptibility test could not be under anesthesia (will not have anesthesia during a certain period before the test is performing (usually pain tests are run in the middle of the experiment, time enough after the immunization and before the terminal experiments, so the anesthesia is not interfere in any case). Because of this, if some animal present high discomfort, the subject will be withdrawn (in any case, this is not what we expect, since these specific tests are performed in chromic models and the humane endpoints are always took into account).

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

☒ 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.

Animals will be monitored daily. Anaesthetics and analgesics will be used to reduce the pain and discomfort of the animals due to some procedures (intra ventricular injection, NCS and EMG). Pain tests are going to be run during the experiment as a part of the model characterization or drug side effects and can be also used as a welfare measurment. No analgesics or anaesthetics will be used in normal procedures like blood extraction or neurological, motor or behaviour assesment/tests.

I. Other aspects compromising the welfare of the animals

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

We can expect adverse effect of the immunization (infections in case of constant infusion through a pump, inflammation in the immunization site, immune system hyperactivity) and the induced disease *per se* due to the pathogenic mechanisms developed (the characteristics symptoms from each disease, i.e. weakness and fatigability in MG and stress, cognitive impairment and seizures in CNS models). Other aspects compromising the welfare of the animals is the *i.p.* injection, which increases the risk of peritonitis and serous fluid production.

There are no adverse effects are expected in relation to the venipuncture or the NCS, neither in the behaviour nor in pain susceptibility tests.

Explain why these effects may emerge.

An unwanted effect could be observed because of immune reaction against the antigen and the permeabilizing agent. The animal will undergo inflammation in the area of injection with possible pain. Pain killers will be administered if necessary. Furthermore, systemic inflammation might occur if the

antibodies start reacting with unspecific endogenous proteins. Many of these antigens have never been used in rodent models and we cannot know how symptoms differ from the human condition.

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

The severity of the induced pathology fluctuates since acute illness can be induced depending on the dose, frequency and duration of the immunization. [redacted]

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.

Humane endpoints will be applied in case of rapid weight loss (>20% within 3 days without any improvement), severe acute inflammation at the immunization point (untreatable by the definition of the veterinarian). The following endpoints are unlikely for completeness we include them since some phenotypes have never been tested before. These includes prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).

We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.

In case of generalized unexpected severe adverse reaction the experiment will be halted.

Indicate the likely incidence.

The severity of the induced pathology fluctuates since acute illness can be induced depending on the dose, frequency and duration of the immunization. All animals will be sacrificed when the disease is more acute, "full blown" to study the pathological mechanisms in a severe situation, which are usually time short time periods. An example of MG passive model, where in an experiment silencing an associated protein to the neuromuscular junction by [redacted] et al., no animal had to be sacrificed before the end of the experiment (max. 72h after immunization). However, an example of the CNS is the work run by Planaguma and colleagues, where the animals had a constant i.v. infusion to reproduce a chronic psychiatric disorder, any of them reach the human endpoints before the end of the experiment (max. 42 days).

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').

All the animals will experience some degree of discomfort. The intermediate analysis of the behavioral, neurologic and/or locomotion tests cause mild to moderate discomfort. Operational procedures generate mild discomfort in 100% cases, however, immunization evokes moderate discomfort. Since all the animals will undergo immunization (except the control group for pain tests), the added discomfort of all tasks is moderate.

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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.

Animals will be sacrificed at the end of the experiment if no adverse events happen.
The sacrifice of the animals will be done according to the analysis requirements, in this case, based on the tissue characteristics. If fixed tissues have to be studied, perfusion is necessary to preserve the organ/tissue in an optimal way. Otherwise, normal sacrifice methods (cervical dislocation) will be used.

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

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

☒ Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
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: 4 Type of animal procedure: Therapy efficacy and side effect in nervous system autoimmune 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.

This appendix describes the application of treatments in a prevention (starting before the disease appears, usually at the same time of the immunization) or therapeutic regimen. The diseases developed are based in nervous system autoimmune disorders, central and peripheral, developed in rodents as described in Appendix 2 and Appendix 3, by active and passive immunization.

The primary outcomes parameters are at two levels: *in vivo* and postmortem analysis.

1. The *in vivo* measures will give information on behavior neurologic and motor status of the animals. The battery of tests used will vary depending on the autoimmune disease model, but the characterization of all the models will be based in behavior (memory, anxiety, etc.), neurologic (electromyography (EMG), nerve conductance speed (NCS)) and locomotion (motor abilities, weakness, fatigue, etc.). This set of tests allows us to better describe the animal status (see brackets in list below).
2. The analysis of the material from the animal models obtained postmortem will be used to assess the biochemical and molecular level of the disease progression and explore the changes after treatment.

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

Therapies modulating the immune system are going to be tested in different nervous system autoimmune disease animal model described in Appendix 1 and 2. Chemical components with autoimmune pathways modulator effect ([redacted] et al., 2010), antibodies targeting molecules of the

nervous system (Stevens et al., under preparation), cells playing a role in the mechanisms and genetic material (DNA or RNA) ([redacted] al., 2009, [redacted] et al., 2016) changing the mechanism of some molecules, essential in the autoimmune diseases, are some examples of the possible therapies that can be applied in this project.
The administration via (s.c. and i.p. are the most common used and electroporation for genetic material using viral or non-viral vehicles), frequency, concentrations and amount of the drugs will be adjusted to the disease model (chronic or acute) and the drug used, based on the reported literature. In case no previous studies have been reported, different administration via and concentrations will be tested in a pilot study to define the optimal parameters (kinetic and chemodynamic properties of the drug will be taken into account) to have the most effective conditions. The treatment efficacy will be assessed to see whether there is a therapeutic window where the treatment is more effective, depending on the disease developed (acute or chronic) and the therapy agent administrated (e.g. in MG rat developed by active immunization, the therapeutic window goes from 4-8 weeks).

To evaluate the efficacy and the side effects of the drugs for the treatment, behavioral, neurological and locomotor tests will be performed in the animal model groups defined in advance and its controls (animals injected with vehicle like saline) (controls of the animal model are also always included to detect the effect of the drug on healthy animals). The outcomes of these tests in the different groups will allow us to assess whether a drug is effective in halting or improving the clinical symptoms of the pathology studied, and therefore provide a stronger translational potential then just analyzing the effect of the drugs using only outcomes from tissue. The animals will undergo the tests regularly (unless they can only be performed once –e.g. EMG) to have different time point data, where a progression of the pathology and drug effect can be studied. Usually, drug studies are performed in chronic disease models to see the progression of the disorder and the effect of the therapy over time and to characterize and had as much information as possible, regular tests are required, like blood samples ([redacted] et al., 2010).

Table 1. Procedures required characterizing neuropsychiatric animal models. Tests are classified according to the main outcome and the character of it: behavior, neurologic and locomotion. A brief description per test and the degree of invasiveness and discomfort has been included.

Procedure/test	**Description**	**Main outcome**	**Example**	**Invasive/ non invasive// discomfort**	**Behavior/ neurologic/ locomotion/ operational**
1. Alternating Y-maze (Hughes RN., 2004)	The animal is introduced in the center of a Y-shaped maze with three white opaque arms. The number of arm entries and the percent of alternations are calculated.	Willingness to explore new environments. Cognitive working memory.	Individuals with cognitive deficits perform less alternation compared to wild type (WT) animals due to the deficit in cognitive working memory.	Non invasive // Mild discomfort (anxiety)	Behavior (cognition)
2. Black and white test box (Costall B. et al., 1989)	The animal is introduced into a box with bright and dark areas. The preference light/dark areas are measured.	Anxiety and exploratory behavior.	The preference by dark areas can be silenced by anxiolytic drugs.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
3. Catwalk (Deacon R.M.J., 2013)	The animal traverses a glass plate voluntarily and the footprints are recorded.	Locomotor activity and nociception.	Minimal physical interference and pain behaviors can be detected and analyzed (different patterns) by this method. Analgesic efficiency can be evaluated.	Non invasive // Mild discomfort	Locomotion

4. Cued and Contextual Fear Conditioning (Curzon P. et al., 2009)	The animal is placed in a box where different expected/unexpected stimulus (light, tone, odor) will be presented. The animal will freeze when an unexpected stimulus is presented. The percentage spent freezing and time for extinction will be measured.	Associative learning and memory (amygdala-hippocampus dependent).	Rodent models with cognitive deficits show less freezing during both cued and context freezing after having been conditioned. Furthermore, they show faster extinction of conditioning compared to WT animals.	Non invasive // Moderate discomfort	Behavior (cognition)
5. Electromyography (EMG) (Osuchowski M.F., et al., 2009)	Different electrodes are located in the paw and in the tail. The time required to transport the electric signal is recorded.	Skeletal muscle electric activity.	In MG rats, EMG is useful to record the degeneration of the muscles and the progression disease.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Neurologic
6. Elevated zero maze test (Kulkarni SK. et al., 2007)	Animals are displaced in an annular or "X" shape elevated platform with open and enclosed quadrants. The time spent in enclosed areas is measured.	Anxiety.	In rodent with cognitive deficits, spent less time in the enclosed arms compared to WT animals, indicating memory deficits.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
7. Encephalography (EEG) (Xie C. et al., 2011)	Brain spontaneous activity is measured and recorded from multiple electrodes placed on the scalp. A video recording is necessary to confirm epileptic seizures.	Electrical brain activity and epileptic seizures.	Epileptic events and encephalopathies can be diagnosed by this method in animal models.	Invasive // Mild discomfort (under anesthesia)	Neurologic
8. Forced swimming tests (Can A. et al., 2012)	The animal is placed in a cylindrical tank with water (the bottom is not reachable and they can not scape from the container). The time spent trying to escape/swim/float is recorded.	Anhedonia, depressive-like behavior	Anti-depressant therapies can be tested using this technique.	Non invasive // Moderate discomfort (anxiety and stress)	Behavior (affect)
9. Horizontal and fixed bars (Deacon RMJ. 2013)	The animal is placed in the middle of a horizontal suspended bar (with different diameters) where they need to walk without falling. In the fixed bars, the different diameters bars are fixed and they need to go from one end of the stick to the base.	Motor function, orientation and coordination	The animal get a composite score: time spent on the bar without falling, capacity to reach the base of the apparatus and the diameter of the bar. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort	Locomotion and neurologic
10. Intubation	Artificial ventilation is	Ensure breading	Electromyography is a	Invasive //	Operational

[redacted] et al., 2015)	provided to the animal through a trachea incision, placing a tube connecting to air flow.		procedure where curare is injected, blocking all muscles making artificial ventilation necessary.	Mild discomfort (under anesthesia, terminal experiment)	
11. Morris Water Maze (Vorhees CV. and Williams MT., 2008)	The rodent is placed in a swimming arena where a submerged platform is located in a strategic place. The time spent to learn will determine the spatial learning and later on the time spent to find the platform will be correlated with the reference memory (the arena is divided by 4 quadrants).	Spatial learning and memory hippocampus dependent. Swimming skills	Rodents with cognitive deficits will spend less time in the target quadrant, because they forgot where it was. The swimming speed can also be measured to determine the locomotion status.	Non invasive // Mild discomfort	Behavior (cognition) and locomotion
12. Nerve conductance speed (NCS) (Osuchowski M. et al., 2009; [redacted] et al., 2015)	Electrodes are placed in the leg and on the tail of the animal. The time required to receive the stimulus from the emitting and the receiving is recorded and correlated with the nervous and muscle status	Nerve damage and neuropathic measurement	Nerve damage can be measured using this technique for example for sciatic nerve impairment. Neuropathy can be tested also as a drug side effect using this technique	Invasive // Mild discomfort (under anesthesia)	Neurologic
13. Object recognition task (Leger M. et al., 2013)	Two similar objects are presented to the animal for the first time and the second time one of them is replaced by a new object. Object exploration duration and habituation times can be analyzed.	Cognition and recognition memory	Animals with cognition impairment will spend more time to recognize the constant and the new object.	Non invasive // Mild discomfort	Behavior (cognition)
14. Olfactory function /Smell threshold concentration test (Yang M. et al., 2009; Katzay A. et al., 2014)	Animals are exposed to increasing concentrations of menthol diluted in mineral oil and the minimal menthol concentration that the mouse responds to is recorded. A mouse response is considered positive when it displays a sharp aversive movement away from the source of smell.	Olfactory function and depression like-behavior	A depressive like behavior is related to decreased olfactory function, so animals with depression will need a stronger olfactory stimulus for positive reaction	Non invasive // Mild discomfort	Behavior (affect)
15. Open Field Test (Bailey KR. And Crawley JN., 2009)	The animal is placed in a square chamber, divided in areas. A recording system will determine the	Locomotion, emotional and anxiety.	Rodent models with cognitive deficits or treated with anxiolytic drugs will show	Non invasive // Mild discomfort (anxiety)	Behavior (affect) and locomotion

	preference of the animal between the periphery and the central areas. In parallel, distance and speed, rare behaviors, grooming, etc. can be evaluated.		reduced time spent in corners due to reduced anxiety.		
16. Parallel rod floor test (Kamens HM and Crabbe JC., 2007)	The animal is placed in a parallel stainless steel rod with a base plate behind. The animal paws are recoded and the number of times they slip through the parallel metal rods and touches the base plate.	Motor coordination (ataxia) and activity.	In rodent with cognitive deficits, locomotion deficits have been observed.	Non invasive // Mild discomfort	Locomotion
17. Perfusion ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is injected in the central venous system with a reagent (PBS or a fixative)/ The liquid is injected in the left atrium and a cut in the right ventricle drain the liquid.	Remove blood and/or preserve the tissue and organs.	Muscles in MG animal models have to be analyzed ab for this, fixed material is necessary because it help to maintain the integrity of the proteins. Brain is another important organ where sometimes the blood has to be removed or the tissue has to be fixed.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Operational
18. Pre-pulse inhibition (PPI) (Powel SB et al., 2009)	The animal receives a weak prestimulus o prepulse that results in the inhibition of the reaction of the animal after a subsequent strong startling stimulus or pulse (usually acoustic or light stimulus).	Anhedonia, depressive-like behavior	PPI has been used primarily in pharmacological animal models to screen putative antipsychotic medications. It is considered to be one of the most promising neurophysiological indexes for translational research in psychiatry (Hidetoshi T et al., 2011).	Non invasive // Mild discomfort	Behaviour (affect)
19. Rack grabbing test ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and a 300g metal rack has to be grabbed repeatedly (5 times with a resting interval of 30s aprox.).	Strength, weakness and fatigability. Depressive-like behaviors.	MG animal models or other muscle impaired models can be graded using this method by the number of repetitions and the time they grab the rack.	Non invasive // Mild discomfort (anxiety)	Locomotion, Behavior (affect)
20. Resident-intruder tests	The animal is placed in a box, first with a sterile	Social interaction, stress and	The social and communication skills	Non invasive // Mild	Behavior (affect) and locomotion

(Koolhaas JM. et al., 2013)	female and later with a male from a non-aggressive strain. Social interaction and stereotypical aggressive behaviors are recorded and later analyzed by the frequency of these activities.	aggressiveness/violence.	and the locomotion can be measured in transgenic mice, where the animals presented more violence. Also the drug effect can be studied in terms of clarity and aggressiveness.	discomfort (anxiety)	
21. Rotarod (Deacon RMJ. 2013)	The animal is placed in a horizontal cylinder (delimitated on the sides). The cylinder rotate at different increasing speeds and the animal have to walk without falling.	Motor function and coordination	The animal get a composite score: ledge test, hind limb clasping, gait, kyphosis (locomotion) and time spent. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort (anxiety)	Locomotion and neurologic
22. Social interaction test (Kaidanovich-Bejilin O. et al., 2011; Sato A. et al., 2013)	The animal is placed in a 3 chamber cage. After habituation, the preference between an empty/known animal/unknown animal is measured. The activity is recorded and number and time spend in each department counted.	Natural sociability and preference novel sociability.	Many neuropsychiatric disorders are characterized by social behavior and recognition disruption. Other locomotion and cognitive parameters can be recorded.	Non invasive // Mild discomfort	Behavior and locomotion (affect)
23. Spatial Y-maze, X-maze and V-maze spontaneous alternation test (Lainiola M. et al., 2014)	The animal is placed in the connection of a 3 arm maze and the exploring capacity is measured by the number of arm entries/alternation. Different objects can be placed at the end of the arms.	Cognitive deficits, memory, anxiety	Transgenic animals or drug effect can be analyzed in the cognitive level using this method, measuring the willingness of rodents to explore new environments. Memory deficits can be measured also by the time spent to recognize the objects.	Non invasive // Mild discomfort (anxiety)	Behavior (affect/ cognition)
24. Sucrose preference/anhedonia test (Strekalova T. et al., 2004)	The rodent is free to choose between a water/sucrose solutions. The % of preference sucrose/liquid consumed is used as criteria (threshold 65%).	Anhedonia and depression-like behaviors	In chronic stress animal model, depression like behavior shows the acuteness of the model with a reduction of sucrose consumption.	Non invasive // Mild discomfort	Behavior (affect)
25. Tail suspension test ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and they need to climb on the top of the hand repeatedly (5 times	Strength, weakness, fatigability. Stress and depressive-	In MG rats, this is a parameter used to observe the acuteness of the disease.	Non invasive // Mild discomfort (anxiety)	Locomotion and behavior (affect)

	with a resting interval of 30s approx.).	like behaviors.	Animals exposed to tail suspension for longer periods daily develop chronic stress.		
26. Venipuncture	A needle is used to puncture an occluded vessel (saphenous vein or submandibular venous sinus). The blood start dripping and after collecting the required amount, you have to press the area to stop the bleeding.	Obtaining a blood sample.	Blood contains a lot of parameters that can be studied in order to monitor autoimmune state in animal disease models like MG, encephalitis, etc. Drug side effects can also be assessed by blood analysis.	Invasive // Mild discomfort	Operational
27. Von Frey and Hargraves (Krzyzanowska A. And Avedaño C., 2012)	The animal is subjected to different thermic and mechanic increasing intensity stimulus. The maximum intensity the animal can withstand without reaction is recorded as a threshold.	Pain susceptibility/ nociception.	Animal models can be tested by this method to assess the susceptibility to pain which reflect their own welfare. Could be an indirect measurement of therapy efficiency or to detect side effects. It also helps to determine the general health.	Non invasive // Moderate discomfort	Behavior (pain)
28. Weight measurement	Animals are placed in a balance and the weight is recorded.	General health.	Weight is a general measurement related with general health, disease progression (for example in MG) and drug side effects.	Non invasive // Mild discomfort	Operational

[redacted]

[redacted]

An example of [redacted]

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

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

All the tests/procedures applied to the animals are well established procedures under a standard protocol (Standardized operational protocol; SOP) which will reduce the bias of the experiment, since in these cases, interaction between the different tasks are already described and we will not make these specific set of tests for the experimental animals. In case it is necessary to include tasks that will affect each other because of the research question, the groups will be divided in subgroups which will follow different sets of tests. On the other hand, another measure to reduce the bias is the design of the tests performed in each experiment, which will be defined by the literature available currently before the performance of the experiments and also based on the expertise of people who has large experience in the behavior test. Under their advice, the tests will be chosen following a stress increasing scale throughout the experiment (in example, an open field will be always performed before a forced swimming test). It is important to mention that all observers (minimum of 2 independent researchers) will be blinded to different groups when performing the tasks.

For this appendix the go/ no-go criteria are the following:

a) Robust and standard: analysis on the different behavior, neurologic and/or locomotion tasks will be performed and *in vitro* analysis as well, definitive the levels of different markers involved in autoimmunity like autoantibodies (specific and unspecific), inflammation molecules and cytokines. For standard animal models, the non-reproducibility of the symptomatology and other markers is a direct no-go criteria, taking into account the variance between experiments. For novel animal models, if no statistically significant differences between the disease and the control group are found, a no-go decision will be followed.
b) Effectiveness: animals administered with the therapeutic drug has to show an improvement of, at least, 10% when compared with the controls. If this is not the case, a no go decision will be followed. After this decision, modifications in the administration route or the doses will be checked and applied if an improvement is available and check again this point.
c) Side effects: in case animals administered with the therapeutic drug suffer an increase of discomfort and/or pain compared to the controls (reaching humane endpoints, section J), a no go decision will be followed. After this decision, modifications in the administration route or the doses will be checked and applied if an improvement is available and check again this point.

For the postmortem study, CSF and blood will be collected for analysis of different molecule levels (e.g. total immunoglobulin levels and specific isotypes and antigen specific immunoglobulin or other factors affected) and cell populations will be studied as well to see how different immune system related cells and molecules can vary due to the disease and the therapy administered. On the other hand, tissues will be collected to be analyzed. Immune related organs – thymus, spleen, lymph nodes, bone marrow –, brain, muscles and other organs which can be affected specifically by the disease developed or the therapy applied – intestines, gut, sciatic nerve, liver, kidney, etc.-. For tissue collection, based on the research question to be answered, different treatments can be applied to the tissue, being necessary to include in some experiments perfusion with different reagents (glutaraldehyde, paraformaldehyde, etc.) of the animals to obtain fixed tissue (e.g. for MG, 2% glutaraldehyde perfused muscles are necessary to study the neuromuscular junction structure). If no perfusions are required, cervical dislocation or CO will be the standard methods to sacrifice the animals.
The techniques we are going to use during this study are: cell based assay (CBA), immunohistochemistry (IHQ), immunofluorescent (IF), radioimmunoassay (RIA), ELISA, Western blot/Dot blot, Flow cytometry, polymerase chain reaction (PCR), electron microscopy can be used and are already operational in our group, based on the literature.

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

For the proposed experiment we will make use of a 2-way ANOVA for analysis of behavioral outcomes. Factors for this ANOVA will be phenotype (disease, control), and treatment (saline, prevention, treatment). By making use of a 2-way ANOVA, we can ensure that the minimum number of animals used, will result in maximum power to determine the contribution of the factors involved.
For analysis of plasma and CSF levels of cells/molecules of interest we will make use of a mixed model analysis. The reason for this is that we can account for any missing values due to failure in assays or missing samples from animals. Fixed factors will be the same as those for the behavioral, neurological and locomotor outcomes, as a covariance structure we will choose compound symmetry.
A power analysis will be performed using the software G*Power, which allows to do power analysis for multi-way ANOVA (and also non -parametric criteria/tests: Friedman test for 3 more matched groups).

As mentioned above, go/no-go decisions will be taken according to the pathology. If no differences are found using a test already reported as a significant difference, the experiment will stop. In case there is no previous report about the test, the experiment will continue as a characterization.

B. The animals

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

For this experiment, rodents, including mice and rats, will be used. Different species, strain, and genetic background (also WT) animals can be included. The specie (3), strain based on the genetic background, gender (2) and age range will be adjusted according to the literature to the most suitable. According to the induced nervous system autoimmune pathology, the onset severity and progression of the disease can vary and also the extension of the model as described in Appendix 1 and Appendix 2.

These experiments will be tested in a preventive (before the animal develop the pathology, starting the treatment at the same time the pathology is induced) and therapeutic (after the pathology is developed in the animal model) regimen.

We distinguish the following groups 6-8 groups (build on phenotype, see below) x 3 to 4 conditions (saline, prevention, treatment, commercial treatment (therapy already tested that acts following the same pathways, as a positive control).

1. Control – saline
2. Control – prevention
3. Control – treatment
4. Control – commercial treatment
5. Disease – saline
6. Disease – prevention
7. Disease – treatment
8. Disease – commercial treatment

9. Control – control (for studies where pain susceptibility tests are required)

Control groups are animals sham immunized or injected with an isotype control antibody and on one hand they are useful to detect the effect of the drug when there is no pathology and to subtract it effects, if present, from the effect on the disease groups taking into account the different setups of administration. On the other hand, there is another control group where the disease animals (and the controls), are injected with saline instead of the drug, which will give us the information of the correct development of the pathology (variances between experiments and animals have been observed) and that nothing is related with the injection procedure.

Using this experimental setup, both the effect of phenotype and the effect of the drug can be analyzed. Furthermore, it might be necessary to test different concentrations of the drug.

Based on theory, we estimate that we will need a maximum of 560 animals per drug study based on previous experience with similar models, for example to show a significance reduction of autoantibody

titers and disease scores in an EAMG model treated with proteasome inhibition. The maximum experiment size will be 140 ((20 animal/study group*4 groups) + (12 animal/ control group*4 groups)=128 animals + 12 animal for the pain test blank = 140), with 3 replication experiments + 1 dose finding study (140*4=560 animals). In this calculation we are taking into account that some animals will have the same conditions but will be sacrificed in different way according to the tissue analysis requirement for every drug and/or the possible necessity to divide the groups in 2 subgroups because of the tasks overlapping. Since we are planning to test 5 possible drugs, this will bring the maximum amount of animals to 2800. However, we expect that the final group size will be significantly smaller after a power calculation. If possible, we will make use of historical controls or control groups generated in previous studies to lower animal numbers and interim analysis to reduce group size.

Summarizing, a total of 2800 animals (1680 rats and 1120 mice) will be included in the "Therapy efficacy and side effect in nervous system autoimmune models" appendix.

All animals will be obtained from a registered supplier or a researcher who have specific animal models in case the registered supplier doesn't have it.

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.

In terms of replacement, these drugs have been validated extensively by *in vitro* experiments to prove their mechanism of action. However, current *in vitro* systems are not able to model all the aspects of these nervous system autoimmune pathologies which are a complex multifactorial disease involving the contribution of many systemic components. While some of these drugs are already used in humans for the treatment of e.g. multiple myeloma, these drugs first have to show beneficial effects in animal models before they can be used in humans. While we expect that the drugs will be beneficial, there might be unforeseen effects which may result in a worsening of the diseases. Therefore, these drugs first have to be evaluated in an animal model.
With the following experiment we want to validate the use of these drugs in different experimental nervous system autoimmune diseases and if we observe a significant beneficial effect the approval for clinical trials will be faster.
In terms of reduction we can limit the amount of animals by investigating several parameters at once like the contribution of the different phenotypes (disrupted BBB, different autoimmune diseases, etc.). Furthermore, because we are planning on testing at least 4 different drugs, we will analyze if it is possible that historical controls can be used, if cohort effects are absent. This will further limit the number of animals. Next, we also choose an animal model with strong behavioral effects. Furthermore, we will perform intermediate analysis to further reduce the number of animals if possible. By doing power calculations for sample size and implementing go/no-go criteria we further limit the amount of animals used.
In terms of refinement, we have chosen to use the most suitable animal model for each pathology (Appendix 2 and 3). This will limit the amount of time that animals are in experiment, and thus experience possible discomfort. Finally, we are going to adapt the accommodation and the care to the need of the animals.

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

We are going to adapt the accommodation and the care to the need of the animals. To limit animal discomfort; anaesthesia and analgesia will be used to reduce the discomfort of the animals during the invasive procedures (see Appendix 2 and Appendix 3). The interaction with the therapeutic agent tested has to be checked before. If the discomfort can be reduced, the subject will withdraw the experiment.

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

☒ 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.

The animals will be checked daily for any sign of pain or discomfort due to treatment or any other experimental procedures hereby described, and if any of these symptoms are detected, analgesics will be applied to relief the suffering.

I. Other aspects compromising the welfare of the animals

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

We can expect adverse effect of the immunization and of the induced disease *per se*, for example, EAMG cause weakness and fatigability and in case of CNS, memory and some neurological and locomotors

impaired abilities can be expected (we can't be sure since no literature is available at the moment), which generate stress as explained more in detail in section K. There are no adverse effects related with the venipuncture or the NCS, neither in the behaviour or pain susceptibility tests.
Animals might experience adverse reaction because of the drugs administered and discomfort may be observed due to the pathology the model develops. These outcomes include increased anxiety, cognitive and neurological impairment locomotor deficits and stereotypical behavior. Furthermore, in some neuropsychiatric animal models, epileptic attacks may be observed. Other aspect compromising the welfare of the animals is the *i.p.* injection, which increases the risk of peritonitis and serous fluid production.

Explain why these effects may emerge.

Although some drugs are already used for other indications, some drugs have never been tested before. Therefore these drugs might cause unwanted side effects that may cause discomfort. Depending on dosage they are potent immunosuppressor. The dose that will be administered will be adjusted to the optimal conditions based on the literature or on dose finding pilot studies. However, unexpected interactions due to the autoimmune pathology cannot be ruled out.

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

Regular monitoring (daily) will prevent and minimize the severity of the welfare since the animals are going to receive pain killer, anesthetics and/or analgesics in case they are necessary (and allowed with the therapeutic agent). The administration site will be checked frequently (the immunization side as well as described in Appendix 2 and Appendix 3) to be sure that no complications appear. If any adverse reactions occur, drug dose will be lowered, or the experiment will be halted. Human endpoints are going to be considered if the animal showed a severe discomfort and euthanasia is going to be applied if the individual reach that status.

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.

Humane endpoints will be applied in case of rapid weight loss (>20% within 3 days without any improvement), severe acute inflammation at the immunization point (untreatable by the definition of the veterinarian). The following endpoints are unlikely for completeness we include them since some phenotypes have never been tested before. These includes prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
So human endpoints will be based on adverse reaction due to treatment. In case of unexpected severe adverse reaction the experiment will be halted.

Indicate the likely incidence.

The incidence will be dependent on the induced disease (Appendix 2 and 3), the character of it: severe or chronic, and on the therapeutic agent and its effects/side effects based also on the dose and the frequency and administration via. Because many combinations can be assessed, is quite hard to determine the incidence value. We estimate the incidence to be extremely low, in the order of 0-5% after a dose finding study. However, for the dose finding study, the incidence cannot be estimated due to the facts that the drug could be a new drug never tested before in vivo and many doses and injections via will be tried or never tested for an specific animal model, where the parameters will be fixed as well.

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').

All the animals will experience some degree of discomfort. The intermediate analysis of the behavioral, neurologic and/or locomotion tests cause mild to moderate discomfort. Operational procedures generate

mild discomfort in 100% cases, however, immunization evokes moderate discomfort. Since all the animals will undergo immunization (except the control group for pain tests), the added discomfort of all tasks is moderate.

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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.

Animals will be sacrificed at the end of the experiment if no adverse events happen. If the animal present severe discomfort or is suffering extremely, according to the human endpoint, this individual will

be sacrificed.

The sacrifice of the animals will be done according to the analysis requirements, in this case, based on the tissue characteristics. If fixed tissues have to be studied, perfusion is necessary to preserve the organ/tissue in an optimal way. Otherwise, normal sacrifice methods (cervical dislocation, CO inhalation) will be used.

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

24 mei 2016

DEC-advies PV 2016-005[redacted]

Preambule;
De DEC-UM verzoekt U eventuele aanvullende vragen rechtstreeks aan de aanvrager te stellen met een afschrift aan de DEC-UM.

A. Algemene gegevens over de procedure

1. **Aanvraagnummer:** 10700

2. **Titel van het project:** *Autoimmunity of the nervous system.*

3. **Titel van de NTS:** *Autoimmuniteit van het zenuwstelsel.*

4. **Type aanvraag:**
   X **nieuwe** aanvraag projectvergunning

5. **Contactgegevens DEC:**
   - naam DEC; *DEC-UM*
   - telefoonnummer contactpersoon; [redacted]
   - e-mailadres contactpersoon; [redacted]

6. **Adviestraject:** (data dd-mm-jjjj):
   X ontvangen door DEC-UM 06-10-2016
   ☐ aanvraag compleet
   X in vergadering besproken 14-10-2016
   ☐ anderszins behandeld
   ☐ termijnonderbreking(en) van / tot
   ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   ☐ aanpassing aanvraag
   ☐ advies aan CCD

7. **Afstemming IvD:**
   - De aanvrager heeft het projectvoorstel afgestemd met de IvD *dd. 06-10-2016*.

8. **Eventueel horen van aanvrager**: ***N.V.T.***

9. **Correspondentie met de aanvrager:**
   - Datum 24-10-2016
   - Gestelde vragen en antwoorden:

De DEC-UM heeft een aantal vragen en opmerkingen:
3.1 Achtergrond
Vragen:
1) Heldere achtergrond, met juiste referenties. De DEC-UM vraagt zich af waardoor patiënten auto-antilichamen aanmaken.
   Graag hier wat meer achtergrond over geven, aangezien dit een belangrijk deel van de pathologie betreft (alhoewel het geen doel van dit PV is).

Antwoord:
Autoimmune diseases are caused by the dysregulation of the immune system against molecules and tissues normally present in the body. It is known that in autoimmune diseases, the immune system is dysregulated and genetic and environmental factors play an important role in the different pathologies. There are several hypothesis that try to explain the origin of autoimmunity but to date only a few like molecular mimicry are more accepted to be at the origin of the deregulation of the immune system.
See Project Proposal > Background.

2) [redacted]

Antwoord:
[redacted]

3) In bijlage 3 onder B komen opeens experimenten aangaande de passage van de placenta "uit de lucht vallen" met de mededeling: "some autoantibodies are targeting fetal antigens, such as AChRy in which case we would use a model of pregnant animals being immunized to study the placenta transfer and effects on the offspring viability and on the fetus/neonate". De DEC-UM wenst hiervoor een nadere toelichting.

Antwoord:
It is known that many molecules can be released from the mother to the fetus (Palmeira et al., 2012). Antibodies are an example of these molecules that can cross the placental membrane and affect the fetus. This has been investigated in autism spectrum disorder, where autoantibodies transferred by the mother can recognize fetal proteins, affecting the baby. However, this reactivity of the mother antibodies was not found in mothers with normal children (Braunschweig D. et al., NeuroToxicology, 2008). This can be also the case of other neuropsychiatric disorders like schizophrenia or bipolar disorder, among others. Because of this, we would like to test the influence of the autoantibodies against neuronal surface antigens, in the offspring and investigate if the antibodies also recognize and affect the fetus.
See Appendix 3 > B. The animals.

3.4 Onderzoeksstrategie
3.4.1
Algemene opmerkingen:
De uitsplitsing in de experimenten is duidelijk, per deeluitsplitsing is de DEC-UM niet helemaal duidelijk wat welke rat ondergaat en welke doelen de verschillende onderdelen dienen.

Reactie:
Goals:
Appendix 1: to prepare the material needed for the generation of animal models, in case of monoclonal antibodies for the passive transfer, and to monitor the development of these animal models. For the last purpose, acetylcholine receptor (AChR) from the denervated rats will be used to monitor the anti-AChR titers in animal models and antibodies –monoclonal and polyclonal– will be used to detect the expression of the antigen in the tissues of the animal models for example in post-mortem studies).

- Rats will be used to extract the brain and process them for immunohistochemistry, that will be used to screen the immunoreactivity of patient sera/ cerebrospinal fluid (CSF) against neuronal proteins.
- Pregnant rats will be used to extract the embryos (the brain will be also extracted and used for the purpose mentioned above).
- Rat embryos (embryogenic day 18, E18) will be used to prepare primary cell culture (hippocampal and cortical cultures), to screen the immunoreactivity of patient sera/CSF against neuronal surface proteins and, in case of an unknown antigen is suspected, immunoprecipitation using the human sera/CSF will be performed to define the pathogenic antigen by mass spectrometry techniques.
- Male rats will be used to obtain large amounts of AChR, after denervation of the sciatic nerve of the hind leg, AChR is necessary for radioimmunoassay when testing the anti-AChR immunoglobulin levels of the EAMG animal model for example.
- Rabbits will be used for the production of polyclonal antibodies against the antigen of interest. This material will be used to perform assays like ELISA or to generate animal models passive transfer.
- Rodents (rats and mice) will be used to produce monoclonal antibodies against the antigen of interest. This material will be used to perform assays like ELISA or for passive transfer animal models.

Appendix 2: generation of active immunization animal models. Models for central and peripheral nervous system (CNS and PNS) will be developed to study the pathogenic mechanisms and the effect of the autoantibodies in a living organism. These animal models can be also used to test new drugs in App.4.

- Rats and mice will be used to develop autoimmune animal models by active immunization after the injection of the antigen of interest which is present in the CNS or PNS

Appendix 3: generation of passive immunization animal models. Models for CNS and PNS will be developed to study the pathogenic mechanisms and the effect of the autoantibodies in a living organism. In this case, the effect of the autoantibodies from a patient can be directly studied in the animal by the infusion of these molecules. These animal models can be also used to test new drugs.

- Rats and mice will be injected (intra venous, intra peritoneal or intra ventricular) with the immunoglobulins against a neuronal antigen from the CNS or PNS(the origin of the antibodies can be from purified patient sera or from a cloned antibody from a patient mainly. Also from other animals immunized with the antigen of interest).

Appendix 4: drug studies. We are going to use the animal models generated in appendix 2 and 3 to study the efficacy of a new or repurposed drug in a specific animal model.
See Project proposal. 3.2. Purpose

Opmerking:
U heeft het over een denervated rat, U gaat ergens tissue gebruiken, wat doet U met de konijnen? De DEC-UM verzoekt U specifieker weer te geven wat waar met welke dieren gebeurt en waarom.
Reactie:
In the denervation part, only rats are going to be used. Rabbits are going to be used to generate polyclonal antibodies as mentioned in appendix 1. All the experiments performed in this appendix have different and independent purposes, but they are essential to perform the appendixes 2 to 4. The generation of AChR will be done by the denervation of sciatic nerve in one of the hind legs. It is known that the amount of receptors increases after the transection (Rochkind S. and Shainberg A., Photomedicine and Laser Surgery, 2013). Since we would like to obtain large amounts of AChR to be used (2 to 4 appendix), we will use male rats for this purpose; the muscles in this specific animals are larger and consequently as well the AChR production. On the other hand, rabbits will be used to generate polyclonal antibodies against any antigen (depending on the antigen injected to the animal). Rats and mice will be used to generate monoclonal antibodies.
See Project proposal . 3.2. Purpose (first aim)
See App1 > A. Experimental approach and primary outcome parameters > Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Vragen:
1) De DEC-UM vraagt zich af welke patiënten (groepen, hoeveel, welke samples, hoeveel worden er getest, hoeveel patiënten zijn nodig) er zijn geselecteerd ?

Antwoord:
We will use brain and primary neuronal cell cultures to screen for the presence of autoantibodies against neuronal antigens in patients with neurological and psychiatric disorders. We will use in house samples already stored in the department, we will keep receiving samples from other hospitals under an ethical approval of rest material and we are currently starting a study already approved by the METC. We will also include samples from control individuals.. Making an estimation, we will use approximately 5000 patient samples, including serum and CSF.
On the other hand, we will also perform animal models by passive transfer. In this case, the antibodies from the patient are going to be administered to the animal. After this step, the animal will be evaluated by different tests such as behavioral, neurological and motor skills.
Results will be compared with those performed by control animals to define the effect of the specific autoantibodies. Later on, post-mortem studies will be performed, analyzing the target organs to further understand the contribution they have in the pathology.
For this purposes, we will use rats and mice as described under appendix 2 and 3.

2) Kunt U voor deeldoel 3 aangeven hoe U de teststoffen selecteert en wat hieraan vooraf gaat?

Antwoord:
Under the subgoal 3 “Efficacy and side effects study of new or repurposed drugs to treat autoimmune diseases of the nervous system” we will use different tests according to the symptomatology of the animal (See Table 2 in appendix 2 and 3).
Based on the literature and the expertise of investigators in our department, we have divided the tasks in 5 different domains that cover all relevant phenotypes in an animal model with an autoimmune disorder affecting the nervous system. The domains are “ affect, cognition, motor, pain and neurologic”. We have included 2 main tests in each domain.

After the first experiment, we will analyze the data and see if any of the domains was more affected when compared with the control. If so, more tests targeting that domain will be performed in the next experimental group to deeply characterize the symptoms.

[redacted]

See Appendix 2 or 3 > A. Experimental approach and primary outcome parameters.

3) Cruciaal lijkt in dit PV het karakteriseren van de eiwitten c.q. antigenen en dat lijkt toch wat eiwitchemie te vooronderstellen. Zijn er eigenschappen (grootte of iets dergelijks) van potentiële eiwitten te noemen, waardoor al enige schifting kan worden gedaan?

Antwoord:

The preselection has been performed based on the autoantibodies already described in patients with psychiatric and neurological disorders. However, there is the possibility that new antigens will be described as potential targets for autoantibodies and because of this, they will fall under the aim of this proposal.

The antigens will be obtained from animals, for example AChR is obtained from Torpedo californica and purified from the electric organ of the fish using an affinity column with cobratoxin, that binds AChR. Other methods like ion exchange and size exclusion can be used to obtain highly concentrated membrane proteins. Another source of antigen will be by protein recombinant production using in vitro models using tags for the purification and/ or other known ligands of the protein.

See Project proposal > Background (last paragraph).

4) Ligt het niet voor de hand uw vraagstellingen eerst eens op te lossen middels toepassing van het EAMG-model en vervolgens de buitenbocht van het onderzoek te nemen?

Antwoord:

The use of the well-established EAMG animal model is mainly to study the effect of new drugs that affect anti-AChR auto-antibody levels and to generate enough preclinical data that allows them to be used in clinical trials for AChR-MG patients (appendix 4). This animal model will not be used in appendixes 2 and 3.

5) Daar U met name geïnteresseerd bent in de pathofysiologie van ionkanaaldefecten ligt gebruik van knockout-muismodellen dan niet in de rede?

Antwoord:

The proteins under study are located in the neuronal surface and they play crucial roles. However, this is not the reason why we still prefer to develop the experiments described for this purpose. Our aim is to study the effect and the mechanisms of action of the autoantibodies against this proteins. Since the effect of them can be different going from the blocking of the protein to the activation of the activity of the same protein, passing to the unfolding of the scavenger proteins, studying the protein through a knock out (KO) will only be relevant to the antibodies with a blocking mechanism of action, and we don't know if this will be the only effect of the autoantibodies.

See project proposal > 3.2 Purpose > Second aim.

3.4.2

Vragen:

1) Veel hangt af van de conservering van de verschillende targets tussen muis/ rat en mens. Kunnen de onderzoekers aangeven voor de verschillende targets die worden genoemd als potentieel belangrijk wat de conservatie is? Antilichamen zijn gericht tegen kleine stukjes eiwit (vaak 8 aa).

Antwoord:

Many groups around the world are currently using rodent proteins to screen the reactivity of human sera against neuronal proteins. This is because of the high homology between the rodent and the human proteins, around 90% (Lancaster E., et al., The Lancet Neurology, 2010, Meizan L, et al., Annals of Neurology, 2009). Fortunately, the pathogenic mechanisms and the role of the autoantibodies from humans can be studied using rodent animal models (see Table 1).

See Project proposal > 3.2 Purpose > First aim

See App1 > A. Experimental approach and primary outcome parameters > Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Table 1. [redacted]

[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]	[redacted]

2) Wat is het nut van "nerve transection" als men is geïnteresseerd in antilichamen? Is dat niet ernstiger dan het matige ongerief dat er in appendix 1 aan wordt toegekend?

Antwoord:

As mentioned before; the nerve transection will be performed for the aim of generating material to follow the progression of the EAMG animal model (see answers to questions 5 and 6). The AChR obtained from the muscle from the transected rats will be used to determine the anti-AChR titers by using a radioimmunoassay (RIA) (see figure 1). We have included humane endpoints according to this experiment, but it is important to mention that any adverse effect involving muscle atrophy has been observed in the past by our team when a bilateral transection of the sciatic nerves was performed.

See Appendix 1 > J. Humane endpoints; A. Experimental approach and primary outcome parameters > Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

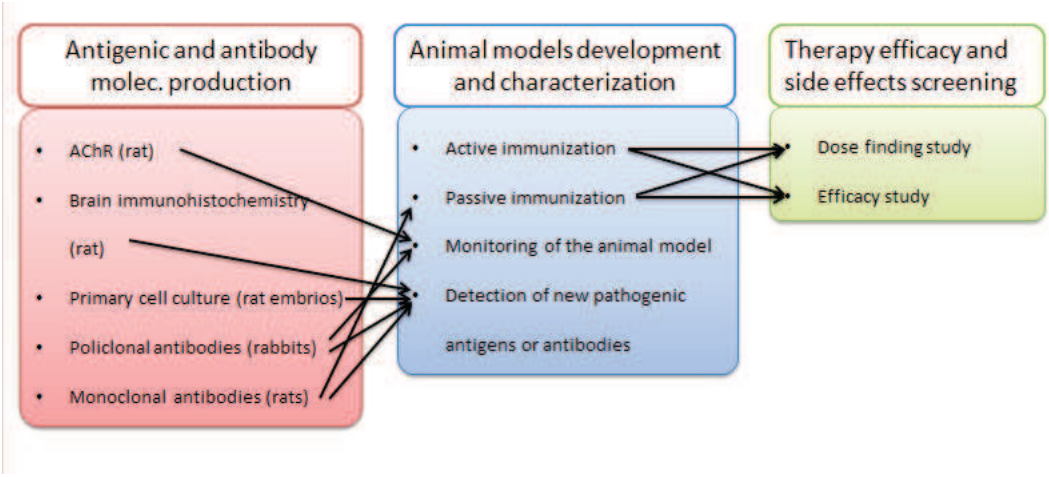

Figure 1. Correlation between the outcomes of each aim proposed in this protocol.

3) Er wordt hier gesteld dat "like before, *in vivo* and *in vitro* characterisation will be performed". *In vitro* wordt nogal eens genoemd in het PV, maar niet nader aangeduid. Eigenlijk roept dit PV de vraag op of er geen *in vitro* screening mogelijk is als tussenstap naar proefdieronderzoek daar ieder potentieel eiwit rücksichtslos in proefdieren lijkt te worden getest. Kunt U de voorafgaande *in vitro* characterisation nader specificeren dan wel ziet U als mogelijkheid tot vermindering bijvoorbeeld gebruik van celcultures om een cytopathogeen effect zichtbaar te maken of met behulp van lymfocyten activatie ervan teneinde een voorscreening van de potentiële eiwitten te doen?

Antwoord:

As soon as we receive the patient material we will search for the pathogenic autoantibodies using in vitro techniques like immunohistochemistry on rat brain and cell based assay, where the protein of interest (the different neuronal antigens in this case, individually), are overexpressed in the surface of the cells. Using this assays we expect to define which of the known antigens is the pathogenic in this specific patient. If we find that none of the autoantibodies are reactive with known antigens, we will perform an immunoprecipitation using rodent material and primary cell cultures from rodents to identify the antigen, additionally we will study the autoantibody effector mechanisms in vitro.

Afterwards, we will perform animal studies, since we would like to understand the mechanism of action of the specific autoantibodies *in vivo*. This is also a requirement to fully demonstrate the autoimmune origin of the disease.

3.4.3

Algemene opmerkingen:

Prima coherentie tussen de verschillende aims. Zou mooi zijn om de verschillende aims nog terug te zien in het schema.

Reactie:

In the project proposal, we have created a low chart schema with all the aims (blue boxes) and the relation between them, where the go/no go criteria are also included. Under the first aim, antigenic and antibody molecules production we will produce molecules to generate and/or characterize the development and progression of known or new nervous system autoimmune animals models and screening and identification of novel autoantibodies targeting molecules in the nervous system in human diseases. For this we will use adult rat tissue, embryonic rat tissue, and rabbits or rodents for antibody generation, as we deeply explain in appendix 1.

Under the second aim we include the next two appendixes, appendix 2 and 3, where we aim to create animal models of autoimmune diseases targeting specific neuronal proteins presents in the CNS or PNS. We will use rodents for this and we will follow an active or a passive immunization to develop the model. For this aim, we will use material obtained from the appendix1/aim 1 or from external sources (commercially available, material from patients, material provided from other researchers, etc.). The monitoring of this animal models will be done using also material obtained from appendix 1. The last aim focused in the drug efficacy study and the side effects it can be found under appendix 4. To test the new or repurposed drug we will use animal models developed under aim 2.

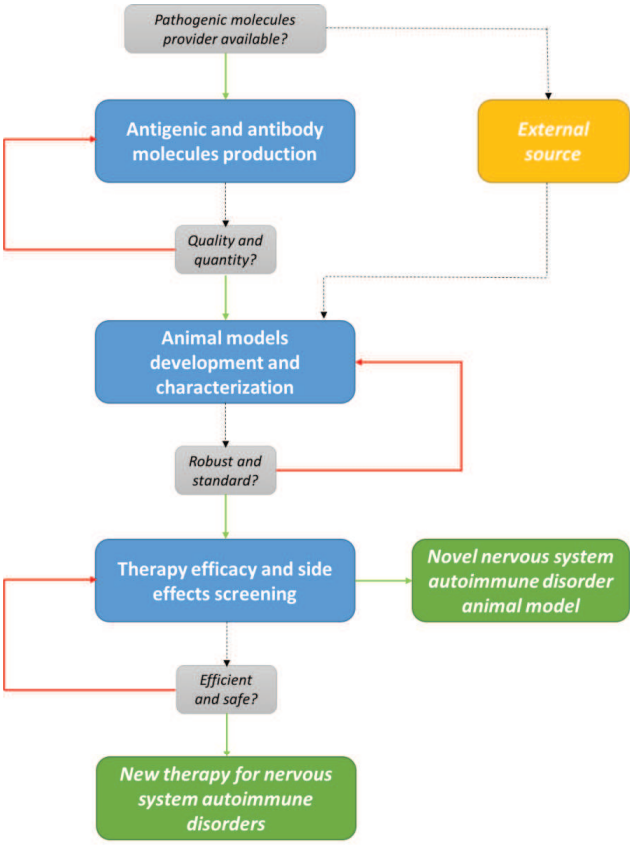

Figure 2. Flow chart showed in project proposal where the aims are described in blue and indirectly correlated with the appendixes and the go/no-go criteria defined.

Vragen:

1) De DEC-UM vraagt of U "A no go criteria will be determined by a not strong enough difference between the animal model and its control or because of non-reproducible data between the different experiments" nader kunt specificeren**.**

Antwoord:

Since the animal models concerning CNS and autoimmunity are not well established yet, we do not know which phenotype we would expect. Because of this constraint, we will follow the procedures performed by authoritative laboratories which already performed similar experiments to characterize other autoantibodies and their targets in the past. If no differences are observed between the model and its control, we will modify some parameters (concentration, administration via, frequency, etc.) to see whether the effect of the antigen/ antibodies can be shown.

See Project proposal > 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.

2) U geeft ondermeer middels een figuur de go/no go-momenten aan. Elders schrijft U (ondermeer in appendix 2) dat met betrekking tot het fenotype het resultaat robuust en standaard moet zijn.
   Vervolgens doet U dit go/no go criterium ernstig verwateren met de opmerking dat dan "changes (e.g. species, gender, route of administration, frequency, dose, adjuvants, etc.) will be implemented to try to generate a robust and standard animal model. Vindt U dit ook geen erg rekbaar go/no go-moment?

Antwoord:
As explained before, most of the animal models, concerning CNS autoimmune disorders, have not been established yet. We will follow the protocols performed previously by competent groups but, due to the differences in the antigen/ autoantibodies, the phenotype can differ. If so, changes will be developed to generate an animal model where the effect of the pathogenic autoantibodies is visible when translated into symptoms. Once we observe differences, we will repeat the experiment using the same conditions to study the reproducibility (robust).
See Project proposal > 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.

3.4.4
Appendix 1

A.

Vragen:

1) Andere weefsels dan hersenen worden als controle mee genomen. Hoe zeker zijn de onderzoekers dat deze weefsels niet ook een positief signaal kunnen hebben en dus goed controle weefsel zijn?

Antwoord:
Brain immunohistochemistry has been used by many groups as a reliable technique to detect immunoreactivity against neuronal antigens in patient sera and/or CSF. This technique allows you to exclude an unspecific reactivity. Next these results can be confirmed by hippocampal primary cell culture afterwards. Finally, to identify the antigen which the autoantibodies are targeting, techniques like cell based assay will be used.
We will test the unspecific signal using the sera/CSF from a healthy control, and we will expect no staining on the brain slices. Using another tissue will not solve the question, since the proteins of interest are highly expressed in brain and not in other tissues.

See Appendix 1 > A. Experimental approach and primary outcome parameters

2) Verwijder het woord "Female" en begin met "Rats" aangezien ook mannetjes ratten gebruikt kunnen worden.

Antwoord:
Thank you for the correction.

3) De DEC-UM vraagt zich af of dit in een deel appendix kan, er worden zulke verschillende handelingen gedaan, volgens de DEC-UM zijn daar de appendices voor bedoeld. Juist om de verschillende handelingen per deelexperiment goed in kaart te brengen? De DEC-UM vindt het nu niet bij elkaar passen, omdat er volledig verschillende zaken gedaan worden.

Antwoord:
We have clarified the text following the comments above and we expect it to be now more clear for the reader. We generate the material to 1) screen and identify the presence of pathogenic or unknown antigens that play a role in neurologic and psychiatric disorders to 2) create animal models and follow the progression of those. In appendix 4, we would like to test drugs to treat the animal models developed in appendixes 2 and 3 described before.

4) Welke alternatieven heeft U overwogen om CFA niet te gebruiken?

Antwoord:
For the generation of monoclonal or polyclonal antibodies Complete Freund's Adjuvant (CFA)is not required., but for animal models it is needed,
See Appendix 1 > A. Experimental approach and primary outcome parameters.

[redacted]
See Appendix 2 > A. Experimental approach and primary outcome parameters.

B.

5) Kunnen de onderzoekers aangeven wat de conservatie is tussen konijn en mens?

Antwoord:
Since rabbits are going to be used to obtain polyclonal antibodies that will only react against the injected antigen (from different origins: human, mouse, rat, etc. depending on our interests), it is not necessary to determine the conservation of the proteins between rabbit and human. We will not inject the animal with CFA (that breaks the tolerance and helps to generate antibodies against self-antigens), despite we will inject Incomplete Freund's Adjuvant (IFA). Therefore, the generated antibodies will only target the foreign (injected) antigen.
See Appendix 1 > A. Experimental approach and primary outcome parameters (at the end).

6) Waar komt het humaan materiaal vandaan? Is de aanvoer zo onzeker? Kan dat niet worden gestroomlijnd?

Antwoord:
[redacted]

This human material will be used in the material obtained in appendix 1 and also to generate passive transfer animal models, described in appendix 3 (autoantibodies cloned or purified) to study in vivo effects and the mechanisms of action. Human material will be indirectly used also in appendix 4 if a therapy is tested in a passive transfer animal model generated using human material.
See Appendix 1 > B. The animals.

7) Dit lijkt niet gebaseerd op de beschikbare teststoffen maar op de dieren?

Antwoord:

[redacted]

See Appendix 1 > B. The animals.

We are sorry, but it is not clear to us, what is meant with “dit” and “teststoffen”. We assume it is either the generation of antibodies or antigenic tissues. Both is not dependent on animals, but on the availability of human materials or commercial antibodies. So, we will generate antibodies in case these are not commercially available to an affordable price (for the purpose of ELISA). The generation of antibodies for passive immunization purpose depends on if the antibody is available from patients (e.g. plasmapheresis material) or not. Also, the generation of antigenic tissue depends on how much human sera we have available to be tested for autoantibodies.

8) Wat gebeurt er met de konijnen?

Antwoord:

Rabbits are going to be used to produce polyclonal antibodies. They will be injected intravenously with the antigen of interest and will develop antibodies against it (but not against self-antigens). After a period of time, when the antibody titer will be in the correct range (tested periodically), we will sacrifice the animals and extract the blood, purify for IgGs and use this material for in vitro experiments.
See Appendix 1 > A. Experimental approach and primary outcome parameters (at the end); B. Animals and D. Replacement, reduction, refinement.

9) Betreft het bilaterale neurectomie?

Antwoord:

We will perform a unilateral transection of the sciatic nerve since we were advice not to do a bilateral transection by the Instituut voor Dierenwelzijn (IvD) due to the disconfort of the animals caused by a bilateral neurectomie. The application of a bilateral transection would have decreased the number of animals by half.
See Appendix 1 > A. Experimental approach and primary outcome parameters

10) Zou gebruik van meer dieren ten aanzien van Ach-receptoroogst neurectomie bij een kleiner aantal dieren kunnen voorkomen?

Antwoord:

No, we could not.. It is known that the amount of receptors is increased under the transection by the sprouting of the nerves to create new muscle terminals (Rochkind S. and Shainberg A., 2013). We would like to obtain large amounts of AChR, so we will use larger animals (male rats) for this purpose. The muscles in this gender of animals are larger and consequently the AChR production will be more prominent.

11) In de 2e alinea 120 inclusief 60 moeders, in de laatste zin, optelsom, lijkt het over 120+60 te gaan.

Antwoord:
We have to include both where, 60 pregnant rats will be used for embryonic primary cell culture and 120 female rats will be used for brain material. Because the time points when we will use these animals can be different (i.e. if we don't need neuronal primary cell cultures at the beginning but we still need brains from adult rats, if we use all the rats included in the proposal for rat brain but not for rat brain and embrios, later on, we will not have rats left for the embrionic neuronal cell culture). Although, as a reduction we will try to include the 60 pregnant female rats into the 120 female rats that we will use for brain tissue to reduce the number of animals. Another source of reduction are the surplus material that we will try to get from other researchers working in the field, brains for example from other groups in the university. We are currently trying to set up the primary cell culture of neurons using neonatal pups, since some groups in the university are presently using these animals and we can get the brain as rest material. However, we have to still include the pregnant rats although the technique is now under revision and the availability of surplus pup issue has not fixed. See B>Animals and D. Replacement, reduction, refinement.

D.

12) Typo: hypridoma = hybridoma

Antwoord:
Thank you for the correction.

13) Waarom worden er zowel polyclonale als monoclonale abs geproduceerd?

Antwoord:
Polyclonal antibodies will be used to label the organs where the antigens are expressed to see for example if the injection of autoantibodies reduces the density of the protein targeted in certain areas. On the other hand, the monoclonal antibodies can be used for the purpose mentioned before, but also to generate passive transfer animal models (Kreye J., et al. Brain, 2016).
See Appendix 1 > A. Experimental approach and primary outcome parameters (at the end).

14) Mogelijk iets noemen over alternatieven CFA?

Antwoord:
For the generation of monoclonal or polyclonal antibodies Complete Freund's Adjuvant (CFA)is not required., but for animal models it is needed,
See Appendix 1 > A. Experimental approach and primary outcome parameters.

[redacted]

See Appendix 2 > A. Experimental approach and primary outcome parameters.

I.

15) U geeft aan “For the last issue, anesthesia or analgesia will be administered to the animal if required”. Brengt U dieren met paralysis posterior 48 uur na de zenuwdoorsnijding wederom onder narcose en voor hoelang?

Antwoord:
After the procedure we will use analgesia, not anesthesia. Anesthesia will be only used during the procedure.
See Appendix 1 > H. Pain and pain relief, I. Other aspects compromising the welfare of the animals.

J.

16) De Paralysis posterior noemt U niet onder de humane eindpunten. Hoe lang tolereert U dit ziektebeeld?

Antwoord:
Animals will be euthanized in case of self-mutilation or autotomy after nerve transection. Pain related with muscle atrophy will be reduced by the use of analgesics if needed. However, it is important to mention that according to our experience, none of the animals followed these behavior, even receiving a bilateral transection.
See Appendix 1 > J. Humane endpoints.

Appendix 2

Algemene opmerking:
Helder uiteen gezet, 2-15 testen per dier, waar is de keuze van 15 op gebaseerd?

Antwoord:
[redacted]
See section A in Appendix 2 and 3.

A.

Vragen:

1) De DEC-UM vraagt zich af wat de onderzoekers bedoelen met “Stability of the antigen”? Als het antigeen niet stabiel is kan dit vals positieve (of negatieve) resultaten opleveren. Hoe controleren de onderzoekers dit om dit te ondervangen?

Antwoord:
With stability of the antigen we mean the half live of the molecule in vivo and the absortion/metabolization using different routes of injection, for example. It is important to mention that all these information will be checked in the literature before, to adjust the strategy to the most suitable.

In some cases, it will be difficult when there is no previous experience in the fiel available. Therefore, in those cases it will be decided based on the outcome results of the animal model (for example, the antibody titers against the specific antigen can be tested using RIA or ELISA).

2) De onderzoekers gebruiken als controle een saline injectie. Dit ondervangt niet de pijn/ het ongerief dat de dieren ondergaan met CFA. Kunnen de dieren hierdoor niet een gedragsverandering ondergaan? Is niet uit te sluiten dat antilichamen die ook buiten de hersenen werken, indirect gedragsveranderingen worden verkregen.

Antwoord:
We will include 3 groups of animals:

1. Animals injected with the antigen + CFA animal model
2. Animals injected with saline + CFA control 1, animal without the disease
3. Animals injected with saline control 2, animal without any immunogen agent injected

With this approach we will be able to know the specific effect of the antigen, by analyzing the effect of the antigen and the effect of the CFA. It is also important to define the effect of CFA since this has never been tested before and maybe it enhances the pain and discomfort sensation in the animals.

See Appendix 2 > A. Experimental approach and primary outcome parameters.

3) Is het te verwachten dat de dieren een gedragsverandering ondergaan vanaf injectie tot aan 6-9 weken behandeling?

Antwoord:
We actually expect the animal to develop a progressive disorder based on the effect of the autoantibodies in the target. As mentioned in EAMG, the effect of the autoantibodies against the AChR is visible after 3-4 weeks from the immunization time point.

4) Is het te verwachten dat de dieren antilichamen ontwikkelen tegen eigen antigenen?

Antwoord:
Yes, it is. This is the purpose of animal models for the active immunization indeed. We intend animals to generate antibodies against the administered antigen, fortunately, the tolerance breakage (CFA injection), could also cross react with the self-antigen and have an effect on it. This has been proved in EAMG and other models (see answer to question 4 in section A, Appendix 1).

5) Met welke gedragstesten brengt u schizofrenie in beeld?

Antwoord:
As mentioned before, since there is not enough information in this field, so based in one study run by Planaguma et al., 2015 and after advice of the behavioural experts we have proposed 5 different domains including:

1) Affect: Elevated zero maze test , sucrose preference test and pre-pulse-inhibition (PPI)
2) Cognition: Alternating Y-maze and object recognition task
3) Motor: Open field and rotarod
4) Pain: Von Frey
5) Neurologic: Electroencephalography (EEG)

After the first pilot, we will analyze the data and see whether any of the domains investigated were affected more than the others. Then we will increase the number of tests into those specific domains to try to characterize them better. The tests will be chosen based on the non-overlapping of outcomes and the non-alteration of the results because of the application of all of them together (bias). In case those interactions between tests are observed at mid-term evaluation of the results, each group will be subdivided and the animals will follow different set of tasks, reducing the bias in this way.
See Appendix 2 and 3 after the table where the tasks are explained.

6) U geeft voor de power analyse slechts een power aan van 0,8 en een significantie van 0,05. Hoe zit het met de andere (uitlees)parameters van de poweranalyse? (idem voor bijlage 3)

Antwoord:
For power calculations on appendix 2 and 3 we will use the effect size as a quantitative measurement of the strength of the phenotype of the animal model. Taking into account the next assumptions: a very large-huge effect size (d=1.20-1.40; Sawilowsky, S. Journal of Modern Applied Statistical Methods, 2009) and a power of 0.80 (Cohen J., Statistical Power Analysis for the Behavioral Sciences, 1988), with an α=0.05 for two-tiled and α=0.025 for one tailed, we will need 9-12 animals per group.
As parameters we will use, in active immunization animal models: IgG titers and weight measurements with some phenotype characteristics (in case of CNS, all the tests proposed in the 5 domains that we will perform in the first pilot) to see whether there are differences or not between the animal model and the controls.

7) Een test voor psychose lijkt te ontbreken.

Antwoord:
We have included the pre-pulse-inhibition in the battery of tests. However, we will use different tests that covers most of the areas afected by neurologic and psychiatric disorders. We will perform test to check the wealfare of the animals regarding affection, cognition, motor habilities, pain sensitivity and neurological impairment. The only area of psychiatric disorders that cannot be covered when compared with the human situation is the presence of hallucinations.
See Appendix 2 and 3 after the table where the tasks are explained.

8) Welke taken voor het bestuderen van schizofrenie-gerelateerde phenotypen worden er meegenomen? Valt het niet aan te bevelen o.a. een pre-pulse-inhibition (PPI) -taak toe te voegen?

Antwoord:
We have included PPI in the affect domain if available in the department. Thanks for your suggestion. See also answer to question 5 in this section.

9) In hoeverre zouden verschillende gedragsveranderingen niet een indirecte maat voor sickness behaviour kunnen zijn? Het valt te verwachten dat een auto-immuunrespons in vele gevallen aspecifieke effecten teweegbrengt die de algehele gezondheidstoestand van het dier beïnvloeden, hetgeen op zijn beurt veranderingen in de voorgenomen gedragstaken kan veroorzaken die los staan van psychopathologie. Hoe denken de onderzoekers hiervoor te corrigeren?

Antwoord:
We expect the symptomatology or phenotype caused by the disease, to be specific due to the injection of the antigen. After the development of autoantibodies against the antigen injected we expect the target tissues to be affected and cause the phenotype. We will be able to at least determine the pure effect of the autoantibodies in the antigen since we will include a control with an injection of saline + CFA and another control with only saline. Also, we will be able to analyze the post-mortem material, which will show the presence of autoantibodies in the target organ and the damage caused by them.

J:

Vragen:

1) Missen bij deze humane eindpunten geen verschijnselen van het zenuwstelsel, want er zijn toch mogelijk ook neurologische complicaties te verwachten, die minder wenselijk zijn ook ten aanzien van het beoogde model? (idem voor bijlage 3 en 4).

Antwoord:
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non-expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
See appendix 2 and 3> humane endpoints.

Appendix 3:

A.

Vragen:

1) De onderzoekers volgen 5 verschillende routes van passieve immunisatie. De DEC-UM vraagt zich af of dit een stapsgewijze aanpak is? Als een manier werkt, moeten dan de andere 4 ook nog volgen?

Antwoord:
We have included different routes of administration due to the diversity of target of the autoantibodies that will be administered to the animal. This parameter will be chosen according to the target, but only one route of administration will be performed. The administration of autoantibodies doesn't need the injection of an adjuvant and luckily it can be directly administered in the organ of target, for example, the brain in case of CNS animal models by intra-ventricular injection or using animal models with a disrupted blood brain barrier (BBB).
See Appendix 3 > A. Experimental approach and primary outcome parameters.

2) U geeft voor de power analyse slechts een power aan van 0,8 en een significantie van 0,05. Hoe zit het met de andere (uitlees)parameters van de poweranalyse?

Antwoord:
For power calculations on appendix 2 and 3 we will use the effect size as a quantitative measurement of the strength of the phenotype of the animal model. Taking into account the next assumptions: a very large-huge effect size (d=1.20-1.40; Sawilowsky, S. Journal of Modern Applied Statistical Methods, 2009) and a power of 0.80 (Cohen J., Statistical Power Analysis for the Behavioral Sciences, 1988), with an α=0.05 for two-tiled and α=0.025 for one tailed, we will need 9-12 animals per group.

As parameters we will use, in active immunization animal models: IgG titers and weight measurements with some phenotype characteristics (in case of CNS, all the tests proposed in the 5 domains that we will perform in the first pilot) to see whether there are differences or not between the animal model and the controls.
In case of passive transfer animal models, weight measurements and the outcome of the tests performed to cover the 5 domains will be taking into account.

J:

3) Missen bij deze humane eindpunten geen verschijnselen van het zenuwstelsel, want er zijn toch mogelijk ook neurologische complicaties te verwachten, die minder wenselijk zijn ook ten aanzien van het beoogde model?

Antwoord:
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non-expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
See appendix 2 and 3> humane endpoints.

Algemene opmerkingen:

- Hier staat CO als dodingsmethode!

Antwoord:
We will use cervical dislocation to sacrifice those animals that will not need to perform a perfusion.

Appendix 4:

A.

Vragen:

1) De DEC-UM vraagt zich af wat bedoeld wordt met “prevention”?

Antwoord:
Prevention is a regimen of treatment where the administration of the drug starts before the disease has been developed, in case of an animal model, specifically in the EAMG, the administration of the medication starts the same week the animals are immunized.

J:

2) Missen bij deze humane eindpunten geen verschijnselen van het zenuwstelsel, want er zijn toch mogelijk ook neurologische complicaties te verwachten, die minder wenselijk zijn ook ten aanzien van het beoogde model?

Antwoord:
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non-expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
See appendix 2 and 3> humane endpoints.

- Datum antwoord 12-12-2016
- Verstrekte antwoorden: *Zie hierboven.*
- De antwoorden hebben wel geleid tot aanpassing van de aanvraag

10. **Eventuele adviezen door experts:** (niet lid van de DEC-UM) ***N.V.T.***

B. Beoordeling (adviesvraag en behandeling)

1. Is het project vergunningplichtig (dierproeven in de zin der wet)? Indien van toepassing, licht toe waarom het project niet vergunningplichtig is en of daar discussie over geweest is. ***Ja***

2. De aanvraag betreft een **nieuwe** aanvraag.

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

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

C. Beoordeling (inhoud)

1. Beoordeel of de aanvraag toetsbaar is en voldoende samenhang heeft.
   Deze aanvraag heeft een concrete doelstelling en kan getypeerd worden als een project. Het is helder welke handelingen en ongerief individuele dieren zullen ondergaan.

   De DEC-UM vertrouwt erop dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en er niet onnodig dieren gebruikt zullen worden. Gezien bovenstaande is de DEC-UM van mening dat de aanvraag toetsbaar is en voldoende samenhang heeft.

2. Geef aan of er aspecten in deze aanvraag zijn die niet in overeenstemming zijn met wet- en regelgeving anders dan de Wod? Denk hierbij aan bijvoorbeeld de Flora en fauna wet en Wet dieren. Indien van toepassing, leg uit om welke aspecten het gaat en waarom hier sprake van is.

   N.v.t., daar dit buiten de taakstelling van de DEC valt overeenkomstig artikel 18a.2.b van de Wod.

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

   Het projectvoorstel heeft inderdaad kenmerken van fundamenteel onderzoek.

   Belangen en waarden

4. Benoem zowel het directe doel als het uiteindelijke doel en geef aan of er een reële relatie is tussen beide doelstellingen.

   Zie antwoord op vraag C5.

5. Benoem de belanghebbenden in het project en beschrijf voor elk van de belanghebbenden welke morele waarden in het geding zijn of bevorderd worden.

   De belangrijkste belanghebbenden in dit fundamenteel wetenschappelijke project, dat gericht is op inzicht in de rol die auto-antilichamen spelen bij ziekten van het zenuwstelsel, zijn de proefdieren, de onderzoekers, de doelgroep/patiënten en hun naasten, en ook de medische wetenschap en de samenleving als geheel.
   Waarden die voor de proefdieren in het geding zijn: De integriteit van de dieren zal worden aangetast door de experimentele handelingen en de gevolgen daarvan en de opoffering aan het eind van de proeven. Het merendeel van de proefdieren zal gering en matig ongerief ondervinden, voor de overige dieren wordt het ongerief geclassificeerd als ernstig.
   Waarden die voor de onderzoekers bevorderd worden: De onderzoekers vergaren kennis over de ziektemechanismen van auto-antilichamen in het zenuwstelsel.
   De onderzoekers zullen medisch-wetenschappelijke kennis verkrijgen die relevant is in het onderzoek naar neurologische en neuropsychiatrische aandoeningen en deze kennis delen met de wetenschappelijke gemeenschap.
   Waarden die voor patiënten bevorderd worden: Uiteindelijk kan meer kennis over de mogelijk auto-immune oorzaak van hun neurologische en neuropsychiatrische symptomen leiden tot verbeterde therapeutische mogelijkheden. Daardoor zou de kwaliteit van leven van deze patiënten en hun naasten verbeterd kunnen worden.
   Groei van medische kennis en mogelijke uitbreiding van het therapeutische arsenaal op een gebied waar daaraan behoefte is, in casu de neurologie en psychiatrie, wordt eveneens bevorderd door het onderhavige onderzoek. Daarom heeft dit onderzoek ook belang voor de samenleving als geheel.

6. Geef aan of er sprake kan zijn van substantiële milieueffecten. Zo ja, benoem deze, leg uit waarom daar sprake van kan zijn en of geef aan of deze effecten afgedekt worden door specifieke wetgeving.

   N.v.t., daar dit buiten de taakstelling van de DEC valt overeenkomstig artikel 18a.2.b van de Wod.

   Proefopzet en haalbaarheid

7. Beoordeel of de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven voldoende gewaarborgd zijn. Licht uw antwoord toe.

   Voor zover de DEC-UM kan beoordelen zijn de kennis en kunde van de onderzoeksgroep adequaat gezien de wetenschappelijke output, de verworven interne- en externe financiering alsmede de aandacht voor de drie V's.

8. Beoordeel of het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en of de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project. Licht uw antwoord toe.

   De DEC-UM is er van overtuigd dat het projectvoorstel aansluit bij recente wetenschappelijke inzichten en geen hiaten bevat die de bruikbaarheid van de resultaten in de weg zullen staan. De voorgestelde experimentele opzet en uitkomstparameters zijn logisch en helder gekozen en sluiten aan bij de aangegeven doelstellingen en de gekozen strategie en experimentele aanpak kunnen naar de mening van de DEC-UM leiden tot het behalen van de doelstelling in het kader van het project.

Welzijn dieren

9. Geef aan of er sprake is van één of meerdere bijzondere categorieën van dieren, omstandigheden of behandeling van de dieren. Beoordeel of de keuze hiervoor voldoende wetenschappelijk is onderbouwd en de aanvrager voldoet aan de in de Wod voor de desbetreffende categorie genoemde beperkende voorwaarden. Licht uw antwoord toe. ***N.V.T.***

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

    De DEC-UM heeft zich ervan verzekerd dat zulks het geval is.

11. Beoordeel of het ongerief als gevolg van de dierproeven realistisch is ingeschat en geclassificeerd, waarbij uitgegaan wordt van de kans op angst, pijn, stress en/of ziekte bij individuele dieren.

    De DEC-UM vertrouwt erop dat de aanvrager al het mogelijke zal doen om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen.

12. Geef aan op welke wijze de integriteit van de dieren wordt aangetast.

    De integriteit van de dieren zal worden aangetast door de experimentele handelingen en de gevolgen daarvan. De dieren worden aan het eind van de proef opgeofferd.

13. Beoordeel of de criteria voor humane eindpunten goed zijn gedefinieerd en of goed is ingeschat welk percentage dieren naar verwachting een humaan eindpunt zal bereiken. Licht uw antwoord toe.

    Naar de mening van de DEC-UM zijn de humane eindpunten zorgvuldig beschreven en is de inschatting van het percentage dieren dat naar verwachting een humaan eindpunt zal bereiken eveneens zorgvuldig beschreven in de projectaanvraag.

14. Beoordeel of de aanvrager voldoende aannemelijk heeft gemaakt dat er geen geschikte vervangingsalternatieven zijn? Onderbouw uw antwoord.

    De DEC-UM is van mening dat de doelstellingen van de proef niet behaald kunnen worden, anders dan met de aangevraagde dieren, daar geschikte vervangingsalternatieven ontbreken, zoals beschreven in onderhavig projectvoorstel.

15. Beoordeel of het aantal te gebruiken dieren realistisch is ingeschat en of er een heldere strategie is om ervoor te zorgen dat tijdens het project met zo min mogelijk dieren wordt gewerkt waarmee een betrouwbaar resultaat kan worden verkregen. Onderbouw uw antwoord.

    Naar de mening van de DEC-UM is het aantal te gebruiken dieren realistisch ingeschat en wel zodanig dat niet meer dan nodig, maar ook niet minder dan nodig dieren worden gebruikt voor het behalen van een betrouwbaar wetenschappelijke resultaat zulks mede gebaseerd op statistische analyse middels een poweranalyse.

16. Beoordeel of het project in overeenstemming is met de vereiste van verfijning van dierproeven en het project zodanig is opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd? Licht uw antwoord toe.

De DEC-UM heeft zich ervan verzekerd dat de aanvrager al het mogelijke zal doen om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen. Hierbij heeft de DEC-UM onder andere de pijnbestrijding en huisvesting in haar beoordeling betrokken.

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

    Voor zover de DEC-UM kan beoordelen zijn de kennis en kunde van de onderzoeksgroep adequaat en mede gezien het daartoe strekkende antwoord van de aanvrager in de projectaanvraag heeft de DEC-UM reden aan te nemen dat onnodige duplicatie achterwege blijft.

    Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Geef aan of dieren van beide geslachten in gelijke mate ingezet zullen worden. Indien alleen dieren van één geslacht gebruikt worden, beoordeel of de aanvrager dat in voldoende mate wetenschappelijk heeft onderbouwd? Geef ook aan welke maatregelen verder zijn getroffen om bij fok of aankoop van dieren het aantal in voorraad gedood te beperken.

    In onderhavige projectaanvraag worden niet uitsluitend dieren van een eenvormig geslacht gebruikt. Alhoewel de DEC-UM vermindering van proefdieren in voorraad gedood toejuicht is zij overigens van mening dat dit aspect met name met de centrale dienst proefdieren en de aanvrager kortgesloten dient te worden daar de DEC niet betrokken is bij de fok en aankoop van proefdieren.

19. Geef aan of dieren gedood worden in kader van het project (tijdens of na afloop van de dierproef). Indien dieren gedood worden, geef aan of en waarom dit noodzakelijk is voor het behalen van de doelstellingen van het project. Indien dieren gedood worden, geef aan of er een voor de diersoort passende dodingsmethode gebruikt wordt die vermeld staat in bijlage IV van de richtlijn. Zo niet, beoordeel of dit in voldoende mate is onderbouwd. Licht dit toe. Indien van toepassing, geeft ook aan of er door de aanvrager ontheffing is aangevraagd.

    Naar de mening van de DEC-UM is dit genoegzaam beschreven in de projectaanvraag door de aanvrager.

20. Indien dieren worden gedood, is adoptie of hergebruik overwogen? Licht toe waarom dit wel/niet mogelijk is.

    Adoptie is ten aanzien van onderhavige aanvraag niet opportuun daar het hier niet handelt om niet-humane primaten, honden, katten of landbouwhuisdieren.

    NTS

21. Is de niet-technische samenvatting een evenwichtige weergave van het project en begrijpelijk geformuleerd?

    Naar de mening van de DEC-UM is zulks het geval.

D. Ethische afweging

1. Benoem de centrale morele vraag.

Rechtvaardigt het verkrijgen van inzicht in de rol die auto-antilichamen spelen bij ziekten van het zenuwstelsel, de opoffering en het ongerief dat de dieren wordt aangedaan in het voorliggende project "Autoimmunity of the nervous system?".

2. Weeg voor de verschillende belanghebbenden, zoals beschreven onder C5, de sociale en morele waarden waaraan tegemoet gekomen wordt of die juist in het geding zijn ten opzichte van elkaar af.

Waarden die voor de proefdieren in het geding zijn: *matig nadeel.*
Waarden die voor onderzoekers bevorderd worden: *substantieel voordeel.*
Waarden die voor de doelgroep bevorderd worden: *matig voordeel.*
Algemeen: *relevante groei van medische kennis.*

De DEC-UM is van mening dat de belangen van de medische wetenschap en de samenleving in het algemeen en van de patiënten en hun naasten binnen het project "Autoimmunity of the nervous system" zwaarder wegen dan de belangen/waarden van de proefdieren. Voor de betrokken proefdieren leiden deze proeven, na voornamelijk gering en matig ongerief, tot de dood. Zij worden door de experimenten en de consequenties daarvan in hun welzijn geschaad. De integriteit van de dieren wordt geschaad door de experimentele handelingen en de gevolgen daarvan, en door de opoffering aan het eind van de proeven.
Indien de doelstellingen bereikt worden, zal dit project echter leiden tot kennis over onderliggende mechanismen van auto-immuun ziekten en auto-antilichamen van het zenuwstelsel karakteriseren bij patiënten. Er zullen nieuwe doelwitten worden geïdentificeerd als basis voor nieuwe therapeutische strategieën.
De verwachting is dat de verworven inzichten op termijn bouwstenen kunnen leveren voor een betere behandeling van patiënten, die lijden aan aandoeningen van het zenuwstelsel. Dergelijke aandoeningen manifesteren zich veelal op relatief jonge leeftijd en resulteren in een aanzienlijke, langdurige en chronische ziektelast en zorgbehoefte, die niet alleen voor de patiënt maar ook voor diens naasten zeer belastend is. Het huidige therapeutische arsenaal is beperkt. Door een verbeterde therapie zou uiteindelijk de kwaliteit van leven verbeterd kunnen worden van belangrijke aantallen patiënten en hun naasten.
Daarnaast is passende zorg voor deze categorie patiënten tijdrovend en kostbaar. Dit onderzoek heeft daarom ook belang voor de samenleving als geheel. Vandaar dat de DEC-UM het onderhavige onderzoek van substantieel belang acht.
Het is aannemelijk dat de doelstelling behaald zal worden. De onderzoekers zullen zoveel mogelijk trachten het lijden van de dieren te beperken d.m.v. anesthesie en pijnbestrijding, waardoor het werkelijke ongerief van de dieren beperkt blijft in relatie tot het te behalen voordeel.

3. Beantwoord de centrale morele vraag.
De DEC-UM beantwoordt de centrale morele vraag:" Rechtvaardigt het verkrijgen van inzicht in de rol die auto-antilichamen spelen bij ziekten van het zenuwstelsel, de opoffering en het ongerief dat de dieren wordt aangedaan in het voorliggende project Autoimmunity of the nervous system?" bevestigend.
Hoewel de DEC-UM de intrinsieke waarde van het dier onderschrijft en oog heeft voor het te ondergane ongerief van de proefdieren, weegt het substantiële belang van dit project naar haar mening zwaarder.

De DEC-UM is van mening dat de voorgestelde experimentele opzet en de uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en dat de gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. De onderzoekers beschikken over de benodigde kennis en technische expertise. Er is geen sprake van duplicatie.
In de gekozen strategie wordt op bevredigende wijze tegemoet gekomen aan de vereisten van vervanging, vermindering en verfijning. De DEC-UM is er van overtuigd dat de aanvrager voldoende maatregelen treft om zowel het ongerief van de dieren als het aantal benodigde dieren tot een minimum te beperken. Er zijn voldoende go/no go momenten voorzien om onnodige dierproeven te vermijden. De DEC-UM is ervan overtuigd dat er geen alternatieven zijn, waardoor deze dierproef met minder ongerief of met minder, dan wel zonder levende dieren zou kunnen worden uitgevoerd.
Op grond van deze overwegingen beschouwt de DEC-UM de voorgestelde dierproeven in het projectvoorstel "Autoimmunity of the nervous system" als ethisch gerechtvaardigd. Derhalve voorziet de DEC-UM het projectvoorstel "Autoimmunity of the nervous system" van een positief advies.

E. Advies

1. Advies aan de CCD
   X De DEC-UM adviseert de vergunning te verlenen onder de voorwaarde dat: indien een fenotype niet gekarakteriseerd wordt als robuust en standaard er dan bij het aanbrengen van eventuele wijzigingen (e.g. species, gender, route of administration, frequency, dose, adjuvants, etc.) in het induceren van dit fenotype, hiertoe toestemming wordt verkregen per modificatie van de IvD, dan wel DEC overeenkomstig de richtlijn meldingen/ wijzigingen.

2. Het uitgebrachte advies is **unaniem** tot stand gekomen.

3. Omschrijf de knelpunten/dilemma's die naar voren zijn gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies zowel binnen als buiten de context van het project.

   N.v.t. Daarenboven is de DEC-UM niet gewoon projectaanvragen buiten de context c.q. haar verantwoordelijkheid en competentie te beoordelen.

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Onze referentie
Aanvraagnummer
AVD107002017808

Bijlagen
2

Datum 3 januari 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted],

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 2 januari 2017. Het gaat om uw project "Autoimmunity of the nervous system". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD107002017808. 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:
3 januari 2017
Aanvraagnummer:
AVD107002017808

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
3 januari 2017
Aanvraagnummer:
AVD107002017808

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10700
Naam instelling of organisatie:	Universiteit Maastricht
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	50169181
Straat en huisnummer:	Minderbroedersberg 4-6
Postbus:	616
Postcode en plaats:	6200 MD MAASTRICHT
IBAN:	NL04 INGB 0679 5101 68
Tenaamstelling van het rekeningnummer:	Universiteit Maastricht

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

Datum:
3 januari 2017
Aanvraagnummer:
AVD107002017808

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:	[redacted]
Functie:	PhD Student
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 februari 2017
Geplande einddatum:	1 februari 2022
Titel project:	Autoimmunity of the nervous system
Titel niet-technische samenvatting:	Autoimmuniteit van het zenuwstelsel
Naam DEC:	DEC-UM
Postadres DEC:	Postbus 616([redacted] 48), 6200 MD Maastricht
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 1.684,-
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:	Maastricht
Datum:	2 januari 2017

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD107002017808

Bijlagen
2

Datum 3 januari 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 3 januari 2017
Vervaldatum: 2 februari 2017
Factuurnummer: 170808

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD107002017808	€ 1.684,00

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.

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Onze referentie
Aanvraagnummer
AVD107002017808

Uw referentie

Bijlagen

Datum 30 januari 2017
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted],

Op 2 januari 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Autoimmunity of the nervous system' met aanvraagnummer AVD107002017808. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

1) U beschrijft in uw aanvraag dat dieren door middel van decapitatie worden gedood. Deze dodingsmethode is in bijlage IV van de richtlijn toegestaan indien onderbouwd waarom geen andere in dezelfde bijlage benoemde methode kan worden gebruikt. We verzoeken u de keuze voor decapitatie te onderbouwen.

2) In bijlage 1 van uw aanvraag meldt u zowel volwassen dieren als embryo's/pups te willen gebruiken. Echter is voor ons niet duidelijk hoe veel embryo's/pups worden ingezet omdat hun aantal in de berekening niet is opgenomen. Hoewel embryo's (in het laatste trimester van zwangerschap) niet in de NVWA registratie worden opgenomen, moeten ze in een projectvergunning worden gemeld. We verzoeken u om het aantal embryo's/pups ook in uw aanvraag aan te geven.

3) In bijlages 2 en 3 van uw aanvraag ontbreekt het percentage dieren dat de humane eindpunten kunnen bereiken. Zou u deze alsnog willen invullen?

4) U geeft in uw aanvraag aan dat het geslacht van de dieren per experiment wordt bepaald. Daarnaast schrijft u dat vrouwelijke Wistar ratten en mannelijke Lewis ratten worden ingezet, dat alleen vrouwelijke konijnen worden ingezet en het geslacht van de muizen wordt niet gespecificeerd. Zou u duidelijker het geslacht van de dieren willen beschrijven en nader kunnen onderbouwen waarom hiervoor wordt gekozen?

5) In de NTS geeft u aan dat 4% van de dieren ernstig ongerief zullen ervaren. Dit is echt niet terug te vinden in de aanvraag. Zou u dit punt willen uitleggen en aanpassen waar nodig?

6) Daarnaast benoemt u 'myasthenis gravis' in uw NTS, een term die waarschijnlijk niet bekend is bij het brede publiek. Zou u deze term willen uitleggen of verwijderen?

7) U beschrijft in uw aanvraag antigenen en antilichamen te willen gebruiken. Kunt u meer informatie geven over de selectiecriteria waarop u de keuzes van antigenen en antilichamen zult baseren?

8) U beschrijft verschillende diermodellen van auto-immuniteit, mede opgezet gebruik makend van nieuwe targets. De CCD vreest hierbij voor ernstig ongerief. Kunt u onderbouwen waarom u er niet van uitgaat dat ernstig ongerief kan optreden?

9) In bijlage 1 beschrijft u drie verschillende experimenten, elk met aparte handelingen en doelstelling. De CCD is van mening dat de samenhang tussen deze experimenten niet voldoende is om ze in dezelfde bijlage te kunnen opnemen. Op deze wijze is moeilijk om de rode draad in deze bijlage te volgen en het splitsen in aparte bijlages zou misschien enkel onduidelijkheden verhelderen. Om de behandeling van uw aanvraag niet verder te vertragen, is de CCD bereid om uw aanvraag administratief te splitsen. Dat houdt in dat u de bijlage niet meer fysiek hoeft te splitsen, maar wel het verschil in leges tussen 4 bijlagen (€1684) en 6 bijlagen (€1970) = €286 dient te voldoen. Indien u hiermee instemt, kunnen we u een factuur voor het verschil in bedrag sturen.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Gebruik hierbij het formulier dat u bij deze brief krijgt indien u uw antwoord per post verstuurt.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat uw aanvraag compleet is. 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.

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

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

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

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

Aanvraagnummer	

2 Bijlagen

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

☐	
☐	
☐	

3 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

Centrale Commissie Dierproeven

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t.a.v. [redacted]
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Onze referentie
Aanvraagnummer
AVD107002017808

Uw referentie

Bijlagen

Datum 30 januari 2017
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Dear members of the Central Commissie Dierproeven,

On January 30, 2017 we have received your feedback on the project permit for animal testing "Autoimmunity of the nervous system "with application number AVD107002017808. In this document you can find the answers and they have been implemented in the specific documents when necessary (all changes are underlined).

We hope to cover all the questions and please, if there is something not clear, don't hesitate to contact us.

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

1) U beschrijft in uw aanvraag dat dieren door middel van decapitatie worden gedood. Deze dodingsmethode is in bijlage IV van de richtlijn toegestaan indien onderbouwd waarom geen andere in dezelfde bijlage benoemde methode kan worden gebruikt. We verzoeken u de keuze voor decapitatie te onderbouwen.

Animals in early stage of development or embryonic stage can only be sacrificed by decapitation (appendix 1) to avoid damaging ther tissue and organs around the neck. Since we are interested in the brains, this is a crucial point. This now has been clarified in appendix 1, section A-1.

2) In bijlage 1 van uw aanvraag meldt u zowel volwassen dieren als embryo's/pups te willen gebruiken. Echter is voor ons niet duidelijk hoe veel embryo's/pups worden ingezet omdat hun aantal in de berekening niet is opgenomen. Hoewel embryo's (in het laatste trimester van zwangerschap) niet in de NVWA registratie worden opgenomen, moeten ze in een projectvergunning worden gemeld. We verzoeken u om het aantal embryo's/pups ook in uw aanvraag aan te geven.

Making an estimation, we will use around 600 embryos in a period of 5 years (10 mothers, with 10 embryos/mother approximately). This now has been clarified in appendix 2, section B-1.

3) In bijlages 2 en 3 van uw aanvraag ontbreekt het percentage dieren dat de humane eindpunten kunnen bereiken. Zou u deze alsnog willen invullen?
In appendix 2, where active immunization animals are developed, we can estimate, based on previous experience in EAMG animal models, that 0-5% of the animals will reach humane endpoints. However, in active immunization targeting other antigens presents in the central nervous system, such an experiment has not been performed before, so we cannot estimate the number of animals that will reach humane endpoints. Based on EAE animal models; where 10% of the animals decrease their body weight more than 20%, which is the most standardized measurement used as humane endpoints, we expect to reach a similar percentage for some antigens (see appendix 2, section J).
In appendix 3, where animal models are developed by passive immunization, there is no literature available about the percentage of animals who had reach humane endpoints in the few experiments that have been performed. Because of this, since the targets are the same than in appendix 2, we expect the same percentage of animals that will reach the humane endpoint. However, it is important to mention, that in this case animals will not build the immune response, since the they will already receive the antibodies and the duration of the experiment will be shorter, we think that the number of animals reaching the humane endpoints will be smaller than 5%. This now has been clarified in see appendix 3, section J.

4) U geeft in uw aanvraag aan dat het geslacht van de dieren per experiment wordt bepaald. Daarnaast schrijft u dat vrouwelijke Wistar ratten en mannelijke Lewis ratten worden ingezet, dat alleen vrouwelijke konijnen worden ingezet en het geslacht van de muizen wordt niet gespecificeerd. Zou u duidelijker het geslacht van de dieren willen beschrijven en nader kunnen onderbouwen waarom hiervoor wordt gekozen?

For polyclonal antibody production, there is a preference for female animals (rats and mice) because females are reported to be group housed more successfully than males because females are more docile and less aggressive in social interaction (Hendriksen and Hau, 2003). This now has been clarified in project proposal, section 3.2, first aim.

For the generation of animals models by active or passive immunization, the selection of the gender will depend on the available literature and the standardized guidelines available for each model. For example, as described in appendix 2, the active immunization model for myasthenia gravis or EAMG is standardized by using Lewis female rats. However, there are other models where the gender is not robustly stablished. An example of it is that, in the literature we have found described no differences between male and female mice in some of the strains, such as C57Bl6, one of the most common strains used in experiments related with our research (Papenfuss T. L., et al., Journal of Neuroimmunology, 2004). This data is in correlation with some studies where mice have been injected with patient autoantibodies, where in some cases both males (Hammer c. Et al., Molecular Psychiatry, 2014; Planaguma J. Et al., Brain, 2015) and females (Castillo-Gomez R. Et al., Annals of Neurology, 2016) where choosen. Because of this, we would like to keep open the decision on the mice gender, since we will adapt it to the most recent literature before starting the experiment. This now has been clarified in project proposal, section 3.2, second aim.

5) In de NTS geeft u aan dat 4% van de dieren ernstig ongerief zullen ervaren. Dit is echt niet terug te vinden in de aanvraag. Zou u dit punt willen uitleggen en aanpassen waar nodig?

We apologize for the mistake in the NTS, we have corrected this. As specified in all the appendixes, all animals will experience some degree of discomfort from mild to moderate due to the behaviour, neurological and locomotor tests and the operational procedures. The immunization with Complete Freud's Adjuvant (appendix 2) and the unilateral nerve transection (appendix 1-section 3) will generate moderate discomfort as well. The animals, due to the procedures or the development of the disorder will not reach severe discomfort, since we will observe them daily and they will be sacrifice before that, according to the described humane endpoints. This now has been corrected in NTS, section 3.5.

6) Daarnaast benoemt u 'myasthenis gravis' in uw NTS, een term die waarschijnlijk niet bekend is bij het brede publiek. Zou u deze term willen uitleggen of verwijderen?

Myasthenia gravis is one of the well-known autoimmune disorders, where the autoantibodies target molecules presents in the neuromuscular junction, such as acetylcholine receptor, resulting in a weakness and fatigability clinical symptomatology. There are many animal models that reproduce the human disorder by active or passive immunization and which have been used to describe the targets, study the pathogenic mechanisms and define the efficiency of new therapies to treat the disease. This now has been clarified in the NTS and project proposal, section 3.1.

7) U beschrijft in uw aanvraag antigenen en antilichamen te willen gebruiken. Kunt u meer informatie geven over de selectiecriteria waarop u de keuzes van antigenen en antilichamen zult baseren?

We are interested in neuropsychiatric disorders with an autoimmune origin. We have based our selection in the literature but this does not mean that other antigens and its autoantibodies will be included in case of been detected. Antigens such as NMDAr, AMPAr, GABABr, GABAAr, VGKC associated proteins among others still unknown have been described as a target in some neuropsychiatric cases but there is still a lot to do to describe the pathogenic mechanisms underlying the disease in these cases and in others, where the target remains still unknown. As mentioned in the relevance of the study, the presence of autoantibodies in neuropsychiatric disorders is not a routine nowadays in most of the clinics around the world and this makes the detection and the description of these targets more difficult. The increase in the knowledge will directly benefit those patients with an autoimmune origin, since they can be treated, whether in another way, a chronic treatment with antipsychotics is administrated. This now has been clarified in project proposal, section 3.2.

8) U beschrijft verschillende diermodellen van auto-immuniteit, mede opgezet gebruik makend van nieuwe targets. De CCD vreest hierbij voor ernstig ongerief. Kunt u onderbouwen waarom u er niet van uitgaat dat ernstig ongerief kan optreden?

All the animal models developed by active or passive immunization will develop a phenotype of mild discomfort that will progress to moderate discomfort with time, since we are working with chronic diseases but also with acute models in some cases. We observe and test the animals daily, which help us to describe the clinical status and the progression of the disorder. When animals reach humane endpoints, they are sacrificed to avoid extra suffering, which will be translated to a severe phenotype. It is important to mention that, due to the chronicity of the

diseases we are working on in most of the cases, this process will not happen suddenly, so the increase of discomfort will be observed, treated in case it is possible, or finished with the sacrifice of the animal. In case of the acute models, the animals will be monitored in a more exhaustive way to identify the presence of any excess of discomfort. This now has been clarified in appendix 2 and 3, section I.

9) In bijlage 1 beschrijft u drie verschillende experimenten, elk met aparte handelingen en doelstelling. De CCD is van mening dat de samenhang tussen deze experimenten niet voldoende is om ze in dezelfde bijlage te kunnen opnemen. Op deze wijze is moeilijk om de rode draad in deze bijlage te volgen en het splitsen in aparte bijlages zou misschien enkel onduidelijkheden verhelderen. Om de behandeling van uw aanvraag niet verder te vertragen, is de CCD bereid om uw aanvraag administratief te splitsen. Dat houdt in dat u de bijlage niet meer fysiek hoeft te splitsen, maar wel het verschil in leges tussen 4 bijlagen (€1684) en 6 bijlagen (€1970) = €286 dient te voldoen. Indien u hiermee instemt, kunnen we u een factuur voor het verschil in bedrag sturen.

We agree with the CCD suggestion and we have divided the appendix 1 in 3 different sections into the same document and labelled them as different appendixes for administrative pourposes:

- Section 1: Brain tissue insolation
- Section 2: Antibody generation
- Section 3: Acetylcholine receptor extraction

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Gebruik hierbij het formulier dat u bij deze brief krijgt indien u uw antwoord per post verstuurt.
Send the adjustments within 14 days to NetFTP. (email)

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat uw aanvraag compleet is. 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.

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

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

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

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

Aanvraagnummer	

2 Bijlagen

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

☐	
☐	
☐	

3 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

Dear member of the Central Commissie Dierproeven,

We would like to thanks for the feedback received on the PV 2016-005: “Autoimmunity of the nervous system”, by application number AVD107002017808.

We have answered the questions in this document and implement the answers in the NTS, the project proposal and the appendixes 1 to 4 when necessary (all changes have been underlined). We have also made references to the documents when this will help to the understanding of the answer.

We would like to know if it is possible for you to check a small modification we have made in the Drug treatment appendix (appendix 4, project PV 2016-005).

In this appendix we have explained the animal experiments we would like to perform to test new or repurposed drugs in different animal models. For this purpose, we have included a maximum of 9 different groups where we have included different therapeutic regimens in control groups (max of 4 * 12 animal/group) and disease groups (max of 4 * 20 animal/group) and an extra group to be able to subtract the effect of the vehicle or sham-treatment. The experiment can be replicated 3 times and an extra experiment for the dose finding is included, if required.

However, [redacted], since although most of the drugs are dissolved in saline; there is the possibility that the drug is dissolved in another vehicle. This modification also affects the group named control – control.

The modification mentioned will not change the number of animals specified in the appendix and it is important to mention that not all the group will be necessary in all the experiments; it will depend on the purpose of each drug therapy experiment.

The changes concerning the last question in appendix 4 have been highlighted in yellow to distinguish from the ones related with the feedback answer. Please let us know if it is possible to rename the groups we would like to have in the animal experiments related with drug testing.

We hope to cover all the questions and please, if there is something not clear, don’t hesitate to contact us.

With many kind regards,

[redacted]

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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
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: 1 Type of animal procedure: Generation of antigenic and antibody sources (section 1, 2 and 3)

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.

This appendix describes the creation and isolation of antigen and antibody rich material using animals to generate and follow the progression of the animal models and the drug screening on them. The same material will be used to identify novel pathogenic antigens in patient's material, which is a positive feedback process to generate novel animal models to describe the pathogenic mechanisms underlying the disease and try therapeutic approaches to treat them. This will include:

- Section 1: Brain tissue insolation: isolation of brain tissue where the antigens are highly concentrated and insolation and culture of cells from different brain areas to obtain primary neuronal cell cultures, astrocytes and microglia
- Section 2: Antibody generation: generation of antibodies by immunization to use them on the development of passive models or other screening purposes
- Section 3: Acetylcholine receptor extraction: isolation of the AChR located in the peripheral nerves and muscle tissue

The primary outcome of this section is based, on one hand, on the generation of diagnostic assays to improve the detection of antibody mediated autoimmunity in central nervous system (CNS), generating rat embryonic primary neuronal cell lines and rat adult brain slices for fluorescent or normal staining, two techniques already well stablished as a diagnostic method in patients with neurologic and psychiatric

impairment. Neuronal antigens are expressed in high densities in the brain when compare with other tissues in the body and have a high interspecies homology (Table 1), so all known autoantibodies causing CNS diseases in humans cross-react with the rat antigen (Lancaster E., et al., 2010, Meizan L, et al., 2009). For this purpose, pregnant female rats and the embryos/pups will be recovered by caesarean section between embryonic day 18 (E18) and birth. The mother will be sacrificed in any case and the brain isolated for use as described above. All these procedures fall into Section 1 of Appendix 1.

For the generation of *in vivo* antibodies, described in section 2 of Appendix 1, we will follow the code of practice for the immunization of laboratory animals. Weekly injections of the antigen of interest are performed, *(s.c.)* to the animal until the immunoglobulin levels reach the peak of expression, around 2-6 weeks after the first injection. The best time point will be determined by testing IgG levels in the blood of the animals (obtained from saphenous vein, every 3-10 days maximum, following the NC3R guidelines for blood sampling).

The use of strong adjuvants (like Complete Freund's Adjuvant, CFA) is required to break tolerance to self-antigens (autoimmunity models by active immunization, see appendix 2). However, for general antibody production (as required here), the use of alternative adjuvants (like incomplete Freund's adjuvant, IFA) will be enough since for this purpose autoimmune reaction is not required. Later on, the animal will be sacrificed and material (e.g. serum and/or splenocytes) will be used for the induction of a passive transfer autoimmune animal model (see appendix 3) and/or to assess the development and the progression of the animal models (see appendix 2 and 3).

On the other hand, the generation of acetylcholine receptor (AChR) is included in this project, specifically in section 3 of Appendix 1, using a denervation technique, where large amounts of the antigens will be generated and then isolated (Rochkind S. and Shainberg A., 2013). The protein (antigen) obtained can be used to perform assays where antibodies from patients or other animal models can be identified and it will also help to generate the active immunization models for AChR (Appendix 2).

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

Section 1: For the neuronal primary cell cultures the animals will be sacrificed (by decapitation, since animals in early stage of development or embryonic stage can only be sacrificed by decapitation to avoid damaging the tissue and organs around the neck. Since we are interested in the brains, this is a crucial point) between embryonic day 18 (retrieved from the uterine horn) and just after birth to create primary cell lines of different cell phenotype. The age depends on the antigen of interest and the expression profile during the certain age. The cells will be used for a diagnostic test to analyse whether autoantibodies against brain antigens are present in serum or cerebrospinal fluid (CSF) of patients with potential central nervous system (CNS) autoimmunity or autoimmune rodent models. Additionally, these cells will be used for *in vitro* assays to study the pathogenic effect of patient autoantibodies on neuronal cells, e.g. internalization of antigen and alteration in ion channel function. Primary cell culture will preferentially be obtained from the unused pups coming from the breeding of the other animal procedures described in this proposal. We are currently working on the setting up of the embryonic cell culture assay, using 1 day born animals, since some groups in the university are regularly working with these animals and we could take the brain as a surplus material.

Rats will be sacrificed to isolate the brain and tissue from other organs (e.g. liver, spleen, etc.) to use as control. The tissue is used for immunohistochemistry (IHC) with patient's IgG in serum or CSF as primary antibody and immunoprecipitation of human autoantibodies. This method is common for the detection of autoantibodies against neuronal antigens in patients, since the homology between the human and the rat proteins is higher than 92% (Lancaster E. 2010; Meizan L. et al., 2009) (Table 1). It is preferred over the use of human post mortem material for different reasons: (a) The antigens are spread over the whole

brain, so the complete brain has to be used for the method, but usually a limited amount of serum/CSF is available to do the staining, which leads to practical difficulties on human brain tissue. (b) The fixation and freezing of the tissue has to be done in a very consistent way to guarantee trustful, replicable diagnostic assays; human tissue is commonly fixed with formaldehyde or freshly frozen, which is not the optimal preparation of the tissue for our purpose. (c) Additionally, the post mortem delay significantly affects the sensitive antigens and introduces variability that might lead to misdiagnosis (false positive or false negative) of autoantibody presence. The animals used for this purpose will mostly be the mothers of the embryos used for creation of primary cell cultures. Yet, during times in which no experiments with embryos are necessary, females from breeding, and males will be also used for the purpose of brain isolation. In case no embryos are required at this period and a denervation experiment is ongoing, brain from the denervated males (section 3) will be also used for this purpose since no gender differences have been identified. It is important to mention that when no primary cell cultures are necessary, brains from rats euthanized in experiments from other researchers at the university will be used in case they are available.

Table 1. [redacted]

[redacted]			

Section 2: For the generation of antibodies in animals, rabbits and mice will be immunized with the antigen of interest. Animals will be tested for raising immunoglobulin levels in serum to determine the number of antigen injections and to sacrifice the animals at the highest peak of antibody levels. Animals will not follow any other kind of tasks and control animals are not required for this purpose. If any of the animals develop a phenotype, as a result of the immunization, the standard humane endpoints (section J) will be applied. For this purpose, the preservation or the homology between the proteins in human and rabbit/rodent is not necessary, since the animal will only develop antibodies against the antigen injected, and we will determine the animal species in advance based on our specific interest. The antibodies generated, in this case polyclonal antibodies, will be used to label the organs where the antigens are expressed, to see, for example, if the injection of autoantibodies reduces the density of that specific protein. On the other hand, monoclonal antibodies can be used for the purpose mentioned before but also to generate passive transfer animal models (Kreye J., et al. Brain, 2016).

Section 3: For the antigen enriched tissue generation of AChR generation, male rats will have a sciatic nerve transection in one of the hind legs. We will use 9 week old (275g) male rats, since those specimens are bigger and the amount of muscle is more abundant due to hormonal differences. In female rats the effect of estrogen on the nervous system would induce a variation that would request higher number of animals per group. Animals will undergo a partial sciatic nerve transection under complete anesthesia in one of the hind legs (unilateral) to reduce the discomfort. Peri and post-operative analgesia will be applied for min 3 days post-operative, prolongation on indication. After the intervention, the nerve will start recovering by sprouting and new neuromuscular junctions will be created, with a higher expression of AChR (Rochkind S. and Shainberg A., 2013). Two weeks later, animals will be brought under

anaesthesia and sacrificed. Afterwards, the receptor derived from rats will be isolated and used in multiple experiments in appendixes 1, 2 and 3. The active immunization models for AChR-MG (immunized using *Torpedo californica* AChR (tAChR)) results in an antibody production targeting not only the tAChR, but also the rat AChR. The amount of antibodies in the serum is a good indicator for the severity of disease in these animals. However, in order to be able to measure the concentration of antibodies against the AChR in the serum of rodents, a radioimmuno assay (RIA) needs to be carried out, wherein the rodent AChR is a necessary reagent. To this end, the animals of this appendix will be euthanized and the carcasses will be then used to extract the antigen.

The methods are operational for some antigens e.g. AChR. For those neuronal antigens that are yet to be identified, the protocol might have to be changed, e.g. the animal might need to be perfused for optimal preparation of the tissue.

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

The number of animals is based on the literature and on previous experience (Elmariah SB et al., 2005, Dalmau J. et al., 2008). As described below we will also make use of rest material of other rodent studies at Maastricht University to minimize the number of animals.

B. The animals

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

Section 1: Rats will be used for this experiment, based on previous experience. Wistar female pregnant rats is the most suitable strain for primary cell culture and brain tissue material based on the literature (however no strain and gender changes has been observed using brains for IHC in our group), while Lewis male rats are the most convenient for sciatic nerve transection due to the biggest size (directly proportional to the muscle and AChR) (e.g. 9 week old Wistar male rat with 275g weight aprox.).

We estimate to use one pregnant rat (~10 embryos/pups) per month, so in a period of 5 years: 60 mothers and 600 embryos or pups.
Animals will only be sacrificed upon need. According to the patient material available, one or two animals at a time will be sacrificed. It is known that 1 animal serves around 100 usable brain slides, which will each allow testing one human sample for antibody presence. We expect to need around 2 animals per month, which means 120 rats over a period of 5 years. However, we are currently already performing this method with tissue from other department of the University which is giving sufficient supply for our current experiments and we already have a biobank of around 15 brains for this purpose, so numbers are likely to be much lower (or even zero) if other departments continue doing similar experiments.
Section 2: For polyclonal antibody generation, animals which can mount a strong immune response are desirable taking into account that the material generated will be larger. For this purpose, there is a preference for female animals because females are reported to be group housed more successfully than males because females are more docile and less aggressive in social interaction (Hendriksen and Hau, 2003). Because of this, female rabbits are the best option for obtaining large quantities of antibodies. We have planned to generate antibodies against a maximum of 6 different antigens and for each we will use a maximum of 15 animals (based on previous experience, which means maximally 90 animals for this

purpose). Additionally, we will use mice for monoclonal antibody generation. In that case, we will use maximal 10 animals per antigen (6 antigens), so a total of 60 mice.

Section 3: For the denervation experiment, an estimated number of 18 adult male Lewis rats will be used based on previous experience. We use adult rats, because we want an antibody repertoire of the adult tissue. In the other appendixes included in this proposal, we will need AChR for about 1500 antibody determinations/experiment with a maximum of 3 repetitions. For each determination, it is necessary to have 150 µl of receptor in solution, which means a total of 675 ml of AChR receptor solution (1500*3*0.150=675ml). In the RIA the standard curve corrects for the concentration of antigen. From an adult rat, 37.5 g of muscle tissue is suited for receptor isolation and taking into account the conversion factor of 600 ml receptor suspension/500g muscle, only 45 ml of receptor solution can be obtained from each individual (600 ml/(500 g/37.5 g/rat)=45 ml/rat). Based on these values, 15 animals will be required to provide enough antigen for the assays proposed. However, experiments in the past have shown that there is a failure rate of 15 % in animals undergoing sciatic nerve transection surgery. Therefore, three additional animals are necessary. In total, we use a max. of 18 male rats.

A total (maximum) of 348 animals (198 rats, 90 rabbits, and 60 mice) are included in this appendix. All animals will be obtained from a registered supplier. These numbers

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.

Concerning refinement, the animals will be housed in groups and with enriched cages to allow species typical behavior. We do not expect any discomfort as the animals will not undergo any procedures in the primary neuronal cell culture and brain slices (section 1) but we expect moderate discomfort in the sciatic nerve resection (section 3). For antibody production (section 2), refinement will take place by using anaesthesia and analgesia for the invasive procedures which minimize the discomfort of the animals.

Concerning generation of antigenic tissue (section 1 and 3), replacement by e.g. cell lines is not possible, because cancer cell lines express many antigens that create background staining of the human antibodies and thereby false positive results.

- For antigen specific tests, some expression systems for e.g. NMDAr, AMPAr, LGI1, and Caspr2 are available and we are using those.
- No cell line exists which overexpresses the receptors/ion channels (complex membrane channel, composed of 4 or more subunits) for this purpose taking into account international guidelines.
- For brain material source, if no embryonic primary cell lines are required, we will ask for rats of other animal studies or we will use, in case a denervation experiment is running, the brains from those animals. Human brain tissue is not an option to use in IHC, since the amount of tissue we can access is low, the quality and fixation of post-mortem human tissue is not of sufficient quality and the size of the human brain is too large, requiring an unrealistic amount of human serum to be tested.

Concerning the production of antibodies (section 2), we will only make use of animals in case there is no hybridoma or expression vector available for producing the antibody targeting the antigen of interest.

- Rabbits are the most suitable specie to generate antibodies *in vivo* but as a replacement Lewis female rat are also a good option based on their enhanced immune response described when generating active immunization models.

Reduction will be considered by using surplus animals when available for the isolation of brains (section 1) and also the brains from the pregnant females and the rat males used for AChR insolation (section 3) but not from the immunized animals (section 2) since the structures can be modified by the presence of antibodies. For the primary cell culture of neuronal tissue (section 1), we are currently trying to set up the technique using 1 day postnatal animals since some groups are regularly working with these animals in the university and they don't need the brain.

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

The well-being of animals will be checked daily and in case of severe suffering or disease (not expected) animals will be sacrificed according to the humane endpoints.

Repetition and duplication

E. Repetition

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

The tissue will be used to characterize autoantibodies in patient serum or CSF or to determine antibody presence in autoimmune models described in all the other appendices. We are analysing sera and CSF of individuals that have not been characterized before. This is assured by communicating with the clincial center that provides the patient material. To our knowledge from conferences and literature there are still many autoantibodies to neuronal antigens still unknown of which the function is not studied.

Accommodation and care

F. Accommodation and care

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

☒ No

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

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

The animals used for antibody production (section 2), will be administered with analgesia in case of discomfort on the injection site. During the nerve transection, animals will be anesthetized (section 3). Additionally, we will use pain killers pre and post-operatively. Optimal procedures will be discussed and decided on with the local IvD/AWB when writing the working protocols.

I. Other aspects compromising the welfare of the animals

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

From immunization with the antigen (section 2), we expect the development of a normal autoimmune reaction including inflammation and starting symptoms of the disease. From the use of incomplete adjuvants, we expect pain in the injection area and a boost of the immune reaction. Due to nerve transection (section 3) we expect locomotion impairments and possibly pain development of the wound after the surgery.

Explain why these effects may emerge.

The only discomfort could arise from complications in the pregnancy (section 1) and sciatic nerve transection (section 3). The transection can impair movement of the hind limbs or paralysis for 48 hours after surgery. For the last issue, analgesia will be administered to the animal if required. Post-operative (also after immunization) analgesia will be applied for min. 3 days post-operative, prolongation upon indication.

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

Animals will be monitored daily. If any adverse reactions occur (not expected) the experiment will be halted. The discomfort generated by antigen immunization (section 2) will be covered with analgesics. The discomfort generated by the nerve transection (section 3) will be diminished with the use of pain killers pre and post-surgery. If animals have problems feeding, we will make use of liquid food or extra rich food to increase the weight or keep it constant. We will also make sure that the animals are able to reach food and water at all times. Humane endpoints will be applied.

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.

Humane endpoints (sections 1 to 3) will be applied in case of rapid weight loss (>20% within 3 days without any improvement), severe acute inflammation at the immunization point (section 2) (untreatable by the definition of the veterinarian) and in case of self-mutation or autotomy after nerve transection (section 3). It is important to mention that according to our experience, none of the animals followed these behavior, even receiving a bilateral transection.
The following enpoints are unlikely, but for completeness we include them since some phenotypes have never been tested before. These include prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).

Indicate the likely incidence.

We expect 0-0.5% of cases will reach the humane endpoints. We do not expect any problems from the pregnant rats to obtain primary cell cultures and brain tissue (section 1). For *in vitro* autoantibody generation (section 2) we do not expect any severe symptomatology since the duration of the experiment will not allow the development of it. For denervated animals (section 3), animals can suffer discomfort due to the paralysis and stress related.

K. Classification of severity of procedures

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

The procedure is a non-recovery procedure for neuronal tissue generation and organ collection (section 1 to 3). The discomfort related with the antibody production (section 2) will be moderate, due to the immunization with incomplete Freund adjuvant or similar and the animals will only suffer an immune reaction with a mild expression of the disease. In case of sciatic nerve denervation (section 3), the discomfort is moderate. The discomfort generated by the nerve transection will be diminished with the use of pain killers pre/post-surgery. Later on the discomfort is based on the paralysis of the hind legs. The animals will be able to move crawling 48h post-surgery and they will be sacrificed after a period of 2 weeks.

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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.

It is necessary to sacrifice the animals to extract cells to start primary cell culture or extract antigenic and antibody tissue.

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

Centrale Commissie Dierproeven

Appendix
Description animal procedures

a) This appendix should be enclosed with the project proposal for animal procedures.
b) A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
c) For more information, see our website (www.centralecommissiedierproeven.nl).
d) 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'.	10700
1.2	Provide the name of the licenced establishment.	University Maastricht
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: 2 — Type of animal procedure: Active immunization in rodents

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.

This appendix describes the development of active immunization rodent models for autoimmune mediated diseases that target antigens in the nervous system. Depending on the antigen that is used to immunize the animals, different nervous system pathologies can be developed. We intend to immunize with neuronal receptors and ion channels leading to autoimmune encephalitis including N-methyl-D-aspartate receptor (NMDAr) and other receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; AMPAr and B gamma-aminobutyric acid receptor; GABABr) or channel associated proteins such as Leucine-rich glioma inactivated 1 (LGI1) or contactin associated protein 2 (Caspr2) which are associated to the voltage gated potassium channel (VGKC), but also antigens in the peripheral nervous system (PNS), expressed in the neuromuscular junction (NMJ), which includes the acetylcholine receptor (AChR), muscarinic kinase receptor (MuSK) and LDL receptor related protein 4 (Lrp4). The injection of any of these antigens (generated in appendix 1 using animals or commercial or recombinant origin) is used to produce an animal model of the disease and/or to study the pathologic progression/mechanisms of the disease and the symptoms induced in the animals. For example, it has been proven in myasthenia gravis (MG), where the injection of MuSK or AChR develop MG and are already well established animal models for autoimmunity (experimental autoimmune myasthenia gravis or EAMG). For central nervous system (CNS) antigens, active immunization has been performed in some pathologies like Multiple sclerosis (MS) where myelin proteins are injected to develop an inflammation model called experimental autoimmune encephalomyelitis (EAE) (Denic A. et al., 2013; Mix E. et al., 2010). This give direct evidence and supports the hypothesis that the injection of the antigen will develop the disorder in an animal model. An autoimmune disease can be either T-cell or B-cell mediated (or a combination of both). To determine what the driving pathogenic force is, the immunized animals will be used to isolate antibodies, T-cell and B-cells and each fraction can subsequently be transferred to naïve animals (passive transfer) to test pathogenicity (which is described in

Appendix 3).

The antigen will be obtained from one of the following sources, depending on the state of research:

a) External sources: electric organ of the *Torpedo californica*, recombinant autoantibodies, *in vitro* systems based on the production of the antigen of interest using eukaryotic cell lines (recombinant DNA in prokaryotic and eukaryotic systems) or any other antigenic SOURCE used to immunize and generate a nervous system autoimmune animal model of interest.
b) In house generation: as described in Appendix 1, the antigenic and antibody molecules have to be, in some cases, in house generated because they are not commercially available. AChR will be obtained from denervated muscle rat (since *in vitro* methods do not produce enough antigen for this purpose), brain expressed antigens will be obtained from rat brains and primary cell cultures from rat embryos and in vitro generated antibodies will be obtained by antigen immunization in rodents. Some of the material will be also used to screen patients' plasma/sera or CSF to identify new pathogenic antigens (e.g. rat brain tissue for immunohistochemistry using patient sera).

The antigen will be administered peripherally (sub cutaneous (s.c.)) to the animal.

The primary outcome is the generation and characterization of autoimmune models targeting the nervous system and will include behavioral (symptomatic), neurological and locomotor tests, followed by post mortem analysis to describe the production of antibodies, T-cells and B-cells and the pathogenic mechanisms of autoantibodies.

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

The immunization varies on the animal model as described above. The frequency is also determined by several parameters like the immunogenicity and stability of the antigen, the severity of the induced disease, etc. with a minimum of a unique immunization at the beginning to a weekly injection until the end of the experiment. Additionally to the antigen, we will use immunogenic reagents to exacerbate the immune reaction since a strong immune response and a break in the tolerance to self-antigens are required to generate an autoimmune model. For this purpose we will use Complete Freund's Adjuvant (CFA) which will be mix with the antigen of interest in an emulsion, injected s.c. in 4 different places in the base of the tail (incomplete FA (IFA) will be used in case of repetitive immunizations (Li, 2004)). CFA will be used to generate the animal models following international guidelines for reproducible and reliable autoimmune models in MG ([redacted] et al., 2015) and other models like collagen-induced arthritis (Brand DD. et al., 2007), autoimmune uveitis (Bansal S. et al., 2015), Tourette syndrome animal model (Horning M. and Lipkin W.I., 2010), experimental autoimmune encephalitis (Randohoff R.M., 2012; Denic A. et al., 2013) or experimental allergic encephalomyelitis (Pachner A.R., 2011) among others. The duration of the experiment varies between 6 to 9 weeks.

a) For the development of a disease, animal models will be generated by a single immunization or weekly immunizations as a maximum frequency, in the same way but studied for up to 9 weeks (chronic model) in case of repetitive immunizations the first immunization will be performed using CFA and the following/s using incomplete FA (IFA) (Li, 2004). During this time, animals will perform different behavioral and neurological tests as described below and will be sacrificed at different time points to analyze pathogenicity and signs of inflammation. Also the blood (obtained from saphenous vein, every 3-10 days maximum, following the NC3R guidelines for blood sampling) will be analyzed for antibody titers, total IgG and antigen specific IgG and leukocyte populations during the disease progression. This will be useful to study progression of the autoimmune disorders induced which can be correlated with behavior, neurologic and locomotor data.

   Control groups are required in all experiments. We will include 3 groups of animals:
   1. Animals injected with the antigen + CFA; animal model
   2. Animals injected with saline + CFA; control 1, animal without the disease
   3. Animals injected with saline; control 2, animal without any immunogen agent injected

With this approach we will be able to know the specific effect of the antigen, by analyzing the effect of the antigen and the effect of the CFA. It is also important to define the effect of CFA since this has never been tested before and maybe it enhances the pain and discomfort sensation in the animals. In case pain is tested, animals without any intervention have to be included to have the basal/physiological level of this measurement.

The motor, neurologic and behavioral battery set up will depend on the nature of the disorders. Also, from each animal model, the batch of tests will be selected to answer the questions as accurately as possible. Every antigen will induce a specific group of symptoms. Some examples of which we expect to study with the associated symptoms are depicted in the table below.

Table 1. [redacted]

[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

[redacted] before. All possible tests have been mentioned in the Table 2 (below). The combination of the tests selected IS disease specific AND is defined according to the pathology and the research question. The choices of the test that we will perform will be based on the latest literature for each pathology reported (Table 1) and on the availability of the tests in our department. It is important to mention that not all the animals in the same experiment will follow the same set of tests, since some of them are incompatible. Some tests have to be performed regularly as described in the Table 2 and others just once, where some others are terminal experiments.

Table 2. Procedures required characterizing neuropsychiatric animal models. Tests are classified according to the main outcome and the character of it: behavior, neurologic and locomotion. A brief description per test and the degree of invasiveness and discomfort has been included.

Procedure/test	**Description**	**Main outcome**	**Example**	**Invasive/ non invasive// discomfort**	**Behavior/ neurologic/ locomotion/ operational**
1. Alternating Y-maze (Hughes RN., 2004)	The animal is introduced in the center of a Y-shaped maze with three white opaque arms. The number of arm entries and the percent of alternations are calculated.	Willingness to explore new environments. Cognitive working memory.	Individuals with cognitive deficits perform less alternation compared to wild type (WT) animals due to the deficit in cognitive working memory.	Non invasive // Mild discomfort (anxiety)	Behavior (cognition)
2. Black and white	The animal is introduced	Anxiety and	The preference by	Non invasive	Behavior (affect)

test box (Costall B. et al., 1989)	into a box with bright and dark areas. The preference light/dark areas are measured.	exploratory behavior.	dark areas can be silenced by anxiolytic drugs.	// Mild discomfort (anxiety)	
3. Catwalk (Deacon R.M.J., 2013)	The animal traverses a glass plate voluntarily and the footprints are recorded.	Locomotor activity and nociception.	Minimal physical interference and pain behaviors can be detected and analyzed (different patterns) by this method. Analgesic efficiency can be evaluated.	Non invasive // Mild discomfort	Locomotion
4. Cued and Contextual Fear Conditioning (Curzon P. et al., 2009)	The animal is placed in a box where different expected/unexpected stimulus (light, tone, odor) will be presented. The animal will freeze when an unexpected stimulus is presented. The percentage spent freezing and time for extinction will be measured.	Associative learning and memory (amygdala-hippocampus dependent).	Rodent models with cognitive deficits show less freezing during both cued and context freezing after having been conditioned. Furthermore, they show faster extinction of conditioning compared to WT animals.	Non invasive // Moderate discomfort	Behavior (cognition)
5. Electromyography (EMG) (Osuchowski M.F., et al., 2009)	Different electrodes are located in the paw and in the tail. The time required to transport the electric signal is recorded.	Skeletal muscle electric activity.	In MG rats, EMG is useful to record the degeneration of the muscles and the progression disease.	Invasive// Mild discomfort (under anesthesia, terminal experiment)	Neurologic
6. Elevated zero maze test (Kulkarni SK. et al., 2007)	Animals are displaced in an annular or "X" shape elevated platform with open and enclosed quadrants. The time spent in enclosed areas is measured.	Anxiety.	In rodent with cognitive deficits, spent less time in the enclosed arms compared to WT animals, indicating memory deficits.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
7. Encephalography (EEG) (Xie C. et al., 2011)	Brain spontaneous activity is measured and recorded from multiple electrodes placed on the scalp. A video recording is necessary to confirm epileptic seizures.	Electrical brain activity and epileptic seizures.	Epileptic events and encephalopathies can be diagnosed by this method in animal models.	Invasive // Mild discomfort (under anesthesia)	Neurologic
8. Forced swimming tests (Can A. et al., 2012)	The animal is placed in a cylindrical tank with water (the bottom is not reachable and they can not scape from the container). The time spent trying to escape/swim/float is recorded.	Anhedonia, depressive-like behavior	Anti-depressant therapies can be tested using this technique.	Non invasive // Moderate discomfort (anxiety and stress)	Behavior (affect)
9. Horizontal and	The animal is placed in	Motor function,	The animal get a	Non invasive	Locomotion and

fixed bars (Deacon RMJ. 2013)	the middle of a horizontal suspended bar (with different diameters) where they need to walk without falling. In the fixed bars, the different diameters bars are fixed and they need to go from one end of the stick to the base.	orientation and coordination	composite score: time spent on the bar without falling, capacity to reach the base of the apparatus and the diameter of the bar. Animals with neurological or locomotor issues will have difficulties and fall soon.	// Mild discomfort	neurologic
10. Intubation [redacted] et al., 2015)	Artificial ventilation is provided to the animal through a trachea incision, placing a tube connecting to air flow.	Ensure breading	Electromyography is a procedure where curare is injected, blocking all muscles making artificial ventilation necessary.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Operational
11. Morris Water Maze (Vorhees CV. and Williams MT., 2008)	The rodent is placed in a swimming arena where a submerged platform is located in a strategic place. The time spent to learn will determine the spatial learning and later on the time spent to find the platform will be correlated with the reference memory (the arena is divided by 4 quadrants).	Spatial learning and memory hippocampus dependent. Swimming skills	Rodents with cognitive deficits will spend less time in the target quadrant, because they forgot where it was. The swimming speed can also be measured to determine the locomotion status.	Non invasive // Mild discomfort	Behavior (cognition) and locomotion
12. Nerve conductance speed (NCS) (Osuchowski M. et al., 2009; [redacted] et al., 2015)	Electrodes are placed in the leg and on the tail of the animal. The time required to receive the stimulus from the emitting and the receiving is recorded and correlated with the nervous and muscle status	Nerve damage and neuropathic measurement	Nerve damage can be measured using this technique for example for sciatic nerve impairment. Neuropathy can be tested also as a drug side effect using this technique	Invasive // Mild discomfort (under anesthesia)	Neurologic
13. Object recognition task (Leger M. et al., 2013)	Two similar objects are presented to the animal for the first time and the second time one of them is replaced by a new object. Object exploration duration and habituation times can be analyzed.	Cognition and recognition memory	Animals with cognition impairment will spend more time to recognize the constant and the new object.	Non invasive // Mild discomfort	Behavior (cognition)
14. Olfactory function /Smell threshold concentration test (Yang M. et al.,	Animals are exposed to increasing concentrations of menthol diluted in mineral oil and the minimal menthol	Olfactory function and depression like-behavior	A depressive like behavior is related to decreased olfactory function, so animals with depression will	Non invasive // Mild discomfort	Behavior (affect)

2009; Katzay A. et al., 2014)	concentration that the mouse responds to is recorded. A mouse response is considered positive when it displays a sharp aversive movement away from the source of smell.		need a stronger olfactory stimulus for positive reaction		
15. Open Field Test (Bailey KR. And Crawley JN., 2009)	The animal is placed in a square chamber, divided in areas. A recording system will determine the preference of the animal between the periphery and the central areas. In parallel, distance and speed, rare behaviors, grooming, etc. can be evaluated.	Locomotion, emotional and anxiety.	Rodent models with cognitive deficits or treated with anxiolytic drugs will show reduced time spent in corners due to reduced anxiety.	Non invasive // Mild discomfort (anxiety)	Behavior (affect) and locomotion
16. Parallel rod floor test (Kamens HM and Crabbe JC., 2007)	The animal is placed in a parallel stainless steel rod with a base plate behind. The animal paws are recoded and the number of times they slip through the parallel metal rods and touches the base plate.	Motor coordination (ataxia) and activity.	In rodent with cognitive deficits, locomotion deficits have been observed.	Non invasive //Mild discomfort	Locomotion
17. Perfusion [redacted] et al., 2010; [redacted] et al., 2015)	The animal is injected in the central venous system with a reagent (PBS or a fixative)/ The liquid is injected in the left atrium and a cut in the right ventricle drain the liquid.	Remove blood and/or preserve the tissue and organs.	Muscles in MG animal models have to be analyzed ab for this, fixed material is necessary because it help to maintain the integrity of the proteins. Brain is another important organ where sometimes the blood has to be removed or the tissue has to be fixed.	Invasive// Mild discomfort (under anesthesia, terminal experiment)	Operational
18. Pre-pulse inhibition (PPI) (Powel SB et al., 2009)	The animal receives a weak prestimulus o prepulse that results in the inhibition of the reaction of the animal after a subsequent strong startling stimulus or pulse (usually acoustic or light stimulus).	Anhedonia, depressive-like behavior	PPI has been used primarily in pharmacological animal models to screen putative antipsychotic medications. It is considered to be one of the most promising neurophysiological indexes for translational research in psychiatry (Hidetoshi T et al.,	Non invasive // Mild discomfort	Behaviour (affect)

			2011).		
19. Rack grabbing test ([redacted]. et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and a 300g metal rack has to be grabbed repeatedly (5 times with a resting interval of 30s aprox.).	Strength, weakness and fatigability. Depressive-like behaviors.	MG animal models or other muscle impaired models can be graded using this method by the number of repetitions and the time they grab the rack.	Non invasive //Mild discomfort (anxiety)	Locomotion, Behavior (affect)
20. Resident-intruder tests (Koolhaas JM. et al., 2013)	The animal is placed in a box, first with a sterile female and later with a male from a non-aggressive strain. Social interaction and stereotypical aggressive behaviors are recorded and later analyzed by the frequency of these activities.	Social interaction, stress and aggressiveness/violence.	The social and communication skills and the locomotion can be measured in transgenic mice, where the animals presented more violence. Also the drug effect can be studied in terms of clarity and aggressiveness.	Non invasive //Mild discomfort (anxiety)	Behavior (affect) and locomotion
21. Rotarod (Deacon RMJ. 2013)	The animal is placed in a horizontal cylinder (delimitated on the sides). The cylinder rotate at different increasing speeds and the animal have to walk without falling.	Motor function and coordination	The animal gets a composite score: ledge test, hind limb clasping, gait, kyphosis (locomotion) and time spent. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort (anxiety)	Locomotion and neurologic
22. Social interaction test (Kaidanovich-Bejilin O. et al., 2011; Sato A. et al., 2013)	The animal is placed in a 3 chamber cage. After habituation, the preference between an empty/known animal/unknown animal is measured. The activity is recorded and number and time spend in each department counted.	Natural sociability and preference novel sociability.	Many neuropsychiatric disorders are characterized by social behavior and recognition disruption. Other locomotion and cognitive parameters can be recorded.	Non invasive //Mild discomfort	Behavior and locomotion (affect)
23. Spatial Y-maze, X-maze and V-maze spontaneous alternation test (Lainiola M. et al., 2014)	The animal is placed in the connection of a 3 arm maze and the exploring capacity is measured by the number of arm entries/alternation. Different objects can be placed at the end of the arms.	Cognitive deficits, memory, anxiety	Transgenic animals or drug effect can be analyzed in the cognitive level using this method, measuring the willingness of rodents to explore new environments. Memory deficits can be measured also by the time spent to recognize the objects.	Non invasive //Mild discomfort (anxiety)	Behavior (affect/ cognition)
24. Sucrose	The rodent is free to	Anhedonia and	In chronic stress	Non invasive	Behavior (affect)

preference/anhedonia test (Strekalova T. et al., 2004)	choose between a water/sucrose solutions. The % of preference sucrose/liquid consumed is used as criteria (threshold 65%).	depression-like behaviors	animal model, depression like behavior shows the acuteness of the model with a reduction of sucrose consumption.	// Mild discomfort	
25. Tail suspension test ([redacted]. et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and they need to climb on the top of the hand repeatedly (5 times with a resting interval of 30s approx.).	Strength, weakness, fatigability. Stress and depressive-like behaviors.	In MG rats, this is a parameter used to observe the acuteness of the disease. Animals exposed to tail suspension for longer periods daily develop chronic stress.	Non invasive //Mild discomfort (anxiety)	Locomotion and behavior (affect)
26. Venipuncture	A needle is used to puncture an occluded vessel (saphenous vein or submandibular venous sinus). The blood start dripping and after collecting the required amount, you have to press the area to stop the bleeding.	Obtaining a blood sample.	Blood contains a lot of parameters that can be studied in order to monitor autoimmune state in animal disease models like MG, encephalitis, etc. Drug side effects can also be assessed by blood analysis.	Invasive // Mild discomfort	Operational
27. Von Frey and Hargraves (Krzyzanowska A. And Avedaño C., 2012)	The animal is subjected to different thermic and mechanic increasing intensity stimulus. The maximum intensity the animal can withstand without reaction is recorded as a threshold.	Pain susceptibility/ nociception.	Animal models can be tested by this method to assess the susceptibility to pain which reflect their own welfare. Could be an indirect measurement of therapy efficiency or to detect side effects. It also helps to determine the general health.	Non invasive // Moderate discomfort	Behavior (pain)
28. Weight measurement	Animals are placed in a balance and the weight is recorded.	General health.	Weight is a general measurement related with general health, disease progression (for example in MG) and drug side effects.	Non invasive //Mild discomfort	Operational

All animals will undergo A behavioral-neurological-locomotor battery adjusted to the induced disease, varying from 2-15 the amount of tests/procedures per animal distributed along the experiment ([redacted]., et al., 2011; Planaguma J. et al., 2015).

An example of CNS passive transfer models with cognitive impairment, is the one performed by Planaguma J. and colleagues, where the animals are characterized using the novel object recognition in open field and V-maze task, sucrose preference/anhedonia test, tail suspension and forced swimming tests, black and white and elevated plus maze tests, resident-intruder test and horizontal and vertical activity assessment. The tasks are performed once to weekly frequency. In our case, we have designed a set of tasks based on 5 domains (specified in Table 2), since the literature is quite terse and the models we will develop might not follow the same pathological mechanisms having different impairments. Based on these domains, we have select a first line tests which will be the first ones applied to characterize the

animal model:

1) Affect: Elevated zero maze test, sucrose preference test and pre-pulse inhibition
2) Cognition: Alternating Y-maze and object recognition task
3) Motor: Open field and rotarod
4) Pain: Von Frey
5) Neurologic: Electroencephalography

After the analysis of the first results, a more specific batch of test will be applied to the specific animal model, focusing on the domains affected. We expect differences in 2-3 domains and we will undertake a maximum of 3 tasks per domain to characterize the animal model in a proper way in those areas where the phenotype is altered (see Table 2). An example of it is the Resident-intruder test or the social interaction, which will introduce more power to an aggressive or abnormal behavior, already observed when the animal is placed in a box with others. The tests will be chosen based on the non-overlapping of outcomes and the non-alteration (bias) of the results because of the application of all of them together. In case those interactions between tests are observed at mid-term evaluation of the results, each group will be subdivided and the animals will follow different set of tasks, reducing in this way the bias.

For the PNS a different battery of task will be applied based on previous experience or recent literature. An example of the batch of tests performed in an active immunization MG model for a drug experiment is [redacted] et al., 2010):

1. Motor: Rack grabbing test and tail suspension test
2. Pain: Von Frey and Hargreaves tests
3. Neurologic: nerve conductance speed and electromyography

Besides the cognition, neurologic and locomotor assessment, some other operational task may be applied to the animal models like general observation (not consider a test/task, since the animals will be checked routinely), venipuncture and weight measurements during the experiment and other terminal tasks like intubation or perfusion at the end of the experiment, depending on the specific purpose of the study.

All the tests/procedures applied to the animals are well established procedures under a standard protocol (Standardized operational protocol; SOP) which will reduce the bias of the experiment, since in these cases, interaction between the different tasks are already described and we will not make these specific set of tests for the experimental animals. In case it is necessary to include tasks that will affect each other because of the research question, the groups will be divided in subgroups which will follow different sets of tests. On the other hand, another measure to reduce the bias is the design of the tests performed in each experiment, which will be defined by the literature available currently before the performance of the experiments and also based on the expertise of people who has large experience in the behavior test. Under their advice, the tests will be chosen following a stress increasing scale throughout the experiment (in example, an open field will be always performed before a forced swimming test). It is important to mention that all observers (minimum of 2 independent researchers) will be blinded to different groups when performing the tasks.

For this appendix the go/ no-go criteria are the following:

1. Animal phenotype
    a) Robust and standard: analysis on the different behavior, neurologic and/or locomotion tasks will be performed as well as *in vitro* analysis defining the levels of different markers involved in autoimmunity like autoantibodies (specific and unspecific), inflammation cells and molecules e.g. plasma cells and cytokines. For standard animal models, the non-reproduction of the symptomatology and other markers is a direct no-go criteria, taking into account the variance between experiments ([redacted] et al., 2015). For novel animal models, if no differences equal or higher than 10% between the disease and the control group are found, a no-go decision will be followed. In this case, the experimental conditions of the model will be studied in detail, and changes (e.g. species, gender, route of administration, frequency, dose, adjuvants, etc.) will be implemented to try to generate a robust and standard animal model. If no differences are found after modifying the strategy, we will report the results as a non-successful or non-viable model for the specific antigen of interest.
    b) Severe: the symptomatology of the animal model will be observed and in case of severe discomfort and/or pain because of the intervention procedures (e.g. immunization), analgesic

will be applied and if even using this the animal keep suffering, a no-go decision will be followed as explained in point a). If animals reach humane endpoints (section J), they will be sacrificed.

2. Test battery

After the analysis of the results of the battery tests, a go/no go decision will followed. In case an animal model shows differences in one of the domains and not in the other, the number of tasks assessing this parameter will be increased and the other test removed from the batch. Moreover, some tests included in Table 2 will be performed in case a strange behavior is observed during routine observation. It is important to mention that, the behavioral battery may change based on results generated from the first groups tested (based on a go/no-go decision after a first characterization in case of CNS active immunization models). For example:

a) When the animal model is new, a characterization of the results in each test will provide an overview of the effect of each antigen specific immunization in the animal model, so a go decision will be followed in this case up to enough reliable data is available.
b) In case of new animal models when a test has been shown (see previous point a)) not to detect differences between groups using a positive and a negative control, it will be discarded from the behavioral battery for this specific animal model (this indicates that the test for that particular model is not useful (no go decision)).
c) When the animal model is well characterized and the results from the test are unexpected, showing no differences between the groups, a no-go decision will be followed.

For the postmortem study, CSF and blood will be collected for analysis of different molecule levels (e.g. total immunoglobulin levels and specific isotypes and antigen specific immunoglobulin or other factors affected) and cell populations will be studied as well to see how different immune system related cells and molecules can vary due to the disease. On the other hand, tissues will be collected to be analyzed. Immune related organs – thymus, spleen, lymph nodes, bone marrow –, brain, muscles and other organs which can be affected specifically by the disease developed – intestines, gut, sciatic nerve, liver, kidney, etc.-. For tissue collection, based on the research question to be answered, different treatments can be applied to the tissue, being necessary to include in some experiments perfusion with different reagents (glutaraldehyde, paraformaldehyde, etc.) of the animals to obtain fixed tissue (e.g. for MG, 2% glutaraldehyde perfused muscles are necessary to study the neuromuscular junction structure).

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

The numbers of animals will be kept to a minimum by collaboration with people who have extensive experience in the generation of autoimmune animal models.
The research strategy will be based on the use of the most informative and descriptive test set for each disease model under the advice of experts in the field, own experience and the use of the literature. We will try to include as many tests as possible taking always into account the bias error and also we will avoid superfluous repetitions. As mentioned above, go/no-go decisions will be taken according to the pathology. If no differences are found using a test already reported to show significant differences, the experiment will be halted. In case there is no previous report about the test, the experiment will continue as a characterization (see above paragraph test battery).

We will use the effect size as a quantitative measurement of the strength of the phenotype of the animal model. Taking into account the next assumptions: a very large-huge effect size (d=1.20-1.40; Sawilowsky, S. Journal of Modern Applied Statistical Methods, 2009) and a power of 0.80 (Cohen J., Statistical Power Analysis for the Behavioral Sciences, 1988), with an α=0.05 for two-tiled and α=0.025 for one tailed, we will need 9-12 animals per group.

As parameters we will use, in active immunization animal models: IgG titers and weight measurements with some phenotype characteristics (in case of CNS, all the tests proposed in the 5 domains that we will perform in the first pilot) to see whether there are differences or not between the animal model and the controls.

B. The animals

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

For this experiment, rodents, including mice and rats will be used. Different species, strain, and genetic background (also WT) animals can be included. The species (3), strain based on the genetic background, gender (2) and age range will be adjusted according to the literature to the most suitable. According to the induced nervous system autoimmune pathology, the onset severity and progression of the disease can vary and also the extension of the model.

- NMJ animal model using AChR is the only model already well established. We will use Lewis female rat, 8-12 weeks of age, are the most susceptible strain and gender for AChR active immunization ([redacted] et al., 2015). Another point in favor to females is that the size of the animal is also important since the amounts of antigen required and the doses of drugs are directly correlated with the weight of them. Other antigens presents in the NMJ can be used to generate the animal models. In these cases, we will use at first the study design used for AChR animal model and the recent literature on the topic (currently there is no information available since we did not define the novel antigen yet).
- For other active immunization models, we will follow the literature available (Denic A. et al., 2013) and in case there is none, we will follow the criteria of other CNS autoimmune models (Planaguma J. et al., 2015, Jones M.V. et al., 2012) or other PNS active immunization models like MG [redacted] et al., 2015). The prevalence of the disorder gender specific in the human population can also be taken into account since the last aim of all research proposes are to reach the human level.

For the animal models of active immunization we will generate a maximum of 5 animal models. For these studies the amount needed depends on the purpose of the animal model (behavior, neurological and/or locomotor description, mechanisms of pathogenicity studies, drug test, new therapies implementation, etc.). We expect a max. of 204 individuals (in example, taking into account 4 groups; disease (20 animal) /healthy (12 animal) * condition "X" /control, in triplicate experiments: ((20*2+12*2)*3=192) an average of the animal number included in each active immunization models, since the genetic background of the animals, the number of conditions to be tested, etc. can be modified (for example, in case pain tests will be included, a non-immunized control group has to be included, so 12 more animals have to be included or the necessity to divide the groups in 2 subgroups because of the tasks overlapping: for example when a task measuring anxiety and another one measuring cognitive impairment cannot be applied in the same animal because one will interfere with the other and the results will not be reliable). In this calculation we have already taken into account that some animals will have the same conditions but will be sacrificed in different way according to the tissue analysis requirement and the high variability existent in active immunization models. In total we will not use more than 1020 animals for active immunization models (204*5=1020 animals).

Summarizing, a total of 1020 animals (816 rats and 204 mice) will be included in the "Active immunization in rodents" appendix.

All animals will be obtained from a registered supplier or a researcher who have specific animal models in case the registered supplier doesn't have it.

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.

Animal models of autoimmune diseases are required to study the *in vivo* pathogenic mechanisms, which will help to understand the procedures underlying the disease. There are no other *in vitro* experimental approaches that can give us this information, because the disease development in this case is very complex and dependent on the interplay of immune system and nervous system. Additionally, we aim to prove that the autoimmune disease can induce neurological and psychiatric symptoms which are difficult to measure in lower organisms.
The number of animals will be keep to the minimum that can give statistical differences by doing power calculations for sample size and implementing go/no-go criteria we further limit the amount of animals used.
Refinement will take place by using anaesthesia and analgesia for the invasive procedures, including pain due to the use of adjuvants during the immunization and the discomfort generated by the development of the pathology. Incomplete FA (IFA) will be used in case that repetitive immunizations are required (Li, 2004).

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

We are going to adapt the accommodation and the care to the need of the animals. To limit animal discomfort; anaesthesia and analgesia will be used to reduce the discomfort of the animals during the invasive procedures, like nerve conductance or for the terminal experiments like electromyography using curare where the animals have to be intubated and receive mechanical ventilation (for more details see Table 1). Animals which are going to follow pain susceptibility test could not be under anesthesia (will not have anesthesia during a certain period before the test is performed). Usually, pain tests are run in the middle of the experiment, enough time after the immunization and before the terminal experiments, so the anesthesia is not interfering in any case. Because of this, if some animal present high discomfort, the subject will be withdrawn (in any case, this is not what we expect, since these specific tests are performed in chronic models and the human endpoints are always taken into account).

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

☒ 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.

Animals will be monitored daily. Anaesthetics and analgesics will be used to reduce the pain and discomfort of the animals due to some procedures (immunization, NCS and EMG). In some cases (e.g. when it is important to asses pain as an outcome parameter), pain tests are going to be run during the experiment as a part of the model characterization and can also be used as a welfare measurment (in MG models pain is expected due to the imunization but in CNS models there is no previous data reporting this).

I. Other aspects compromising the welfare of the animals

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

We can expect adverse effect of the immunization and of the induced disease *per se*, for example, EAMG cause weakness and fatigability (including problems with food intake and difficulties to breathe or the appearance of elephant teeth) and in case of CNS, memory and some neurological and locomotors impaired abilities can be expected, which generate stress as explained more in detail in section A.

There are no adverse effects are expected in relation to the venipuncture or the NCS, neither in the behaviour nor in pain susceptibility tests.

Explain why these effects may emerge.

An unwanted effect could be observed because of immune reaction against the antigen and the adjuvant. The animal will undergo inflammation in the area of injection with possible pain. Pain killers will be administered if necessary. Furthermore, systemic inflammation might occur if the antibodies produced by the animals start reacting with endogenous proteins. Many of these antigens have never been used in rodent models and we cannot know how symptoms differ from the human disease onset and progression.

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

Regular monitoring will prevent and minimize the discomfort since the animals are going to receive pain killer, anesthetics and/or analgesics in case they are necessary. The immunization site will be checked the days after the intervention to be sure that no complications appear. Human endpoints will be defined specific for the pathology studied and in case the animal shows a severe discomfort, euthanasia will be performed if the individual reach that status.

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.

Humane endpoints will be applied in case of rapid weight loss (>20% within 3 days without any improvement–some autoimmune models have a transient acute phase of the disease which

spontaneously recovers), severe acute inflammation at the immunization point (untreatable by the definition of the veterinarian). The following endpoints are unlikely for completeness we include them since some phenotypes have never been tested before. These includes prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
In case of generalized unexpected severe adverse reaction the experiment will be halted.

Indicate the likely incidence.

The severity of the induced pathology fluctuates since acute or chronic illness can be induced depending on the dose, frequency and duration of the immunization (Lennon V. et al., 1975; [redacted] et al., 2015).

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').

All the animals will experience some degree of discomfort. The intermediate analysis of the behavioral, neurologic and/or locomotion tests cause mild to moderate discomfort. Operational procedures generate mild discomfort in 100% cases, however, immunization evokes moderate discomfort (single immunization with CFA and repetitive immunizations with IFA). Since all the animals will undergo immunization (except the control group for pain tests, which will not have CFA injection), the added discomfort of all tasks is moderate.

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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.

Animals will be sacrificed at the end of the experiment.
The sacrifice of the animals will be done according to the analysis requirements, in this case, based on the tissue characteristics. If fixed tissues have to be studied, perfusion is necessary to preserve the organ/tissue in an optimal way.

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

Centrale Commissie Dierproeven

Appendix
Description animal procedures

1) This appendix should be enclosed with the project proposal for animal procedures.
2) A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
3) For more information, see our website (www.centralecommissiedierproeven.nl).
4) 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'.	10700
1.2	Provide the name of the licenced establishment.	University Maastricht
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: Passive immunization in rodents

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.

This appendix describes the development of passive immunization rodent models for autoimmune mediated diseases that target antigens in the nervous system.

[redacted]

[redacted]

The primary outcome is the generation of autoimmune models of the nervous system to study the pathogenic mechanisms of the autoimmune disorders affecting the nervous system. The measures will include behavioral (symptomatic), neurological and locomotor tests, followed by post mortem analysis to describe the pathogenic mechanisms of the autoimmune diseases.

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

The immunization varies on the animal model as described above. The frequency is also determined by several parameters like the immunogenicity and stability of the antibodies, the severity of the induced disease, etc. with a minimum of a unique immunization at the beginning to a constant perfusion of the antibody until the end of the experiment. For peripheral injections, i.p. injections are the most common ones unless the antibody target is highly expressed in the peritoneal cavity, being then i.v. administration the optimal. For intrathecal injection, a constant intra-ventricular infusion via a minipump is optimally used. In shorter experiments (<1 week) only one or two intra-ventricular injections would be considered. In case the antibody has to reach the CNS and the injection is peripheral, BBB permeabilization reagents will be used, together with the antibodies, as described above. The immunization site will be checked the days after the intervention to be sure complications will not appear.

Control groups are required in all experiments. We will include a control without the disease (a non-pathogenic antibody is going to be injected instead of the pathogenic antibodies). The control group will be treated equally as the study group, meaning that permeabilize reagents will be also injected to control group if required. In case the study is based on the capacity of the antibody to penetrate the BBB, the study group is composed by 2 groups (antibody with and without the permeabilization), 2 control groups will be required (antibody without permeabilization and permeabilization without antibody).

The motor, neurologic and behavioral battery set up will depend on the nature of the disorders. Also, from each animal model, the batch of tests will be selected to answer the questions as accurately as possible. Every antibody will induce a specific group of symptoms. Some examples of which we expect to study with the associated symptoms are depicted in the table below.

Table 1. [redacted]

Animal procedures selected will depend on the purpose of the study as mentioned before. All possible tests have been mentioned in the Table 2 (below). The combination of the tests selected disease specific is defined according to the pathology and the research question. All the choices about the test that we will perform will be based on the latest literature for each pathology reported (Table 1) and on the availability of the tests in our department. It is important to mention that not all the animals in the same experiment will follow the same set of tests, since some of them are incompatible. Some tests have to be performed regularly as described in the Table Y and others just once, where some others are terminal experiments.

Table 2. Procedures required characterizing neuropsychiatric animal models. Tests are classified according to the main outcome and the character of it: behavior, neurologic and locomotion. A brief description per test and the degree of invasiveness and discomfort has been included.

Procedure/test	**Description**	**Main outcome**	**Example**	**Invasive/ non invasive// discomfort**	**Behavior/ neurologic/ locomotion/ operational**
1. Alternating Y-maze (Hughes RN., 2004)	The animal is introduced in the center of a Y-shaped maze with three white opaque arms. The number of arm entries and the percent of alternations are calculated.	Willingness to explore new environments. Cognitive working memory.	Individuals with cognitive deficits perform less alternation compared to wild type (WT) animals due to the deficit in cognitive working memory.	Non invasive // Mild discomfort (anxiety)	Behavior (cognition)
2. Black and white test box (Costall B. et al., 1989)	The animal is introduced into a box with bright and dark areas. The preference light/dark areas are measured.	Anxiety and exploratory behavior.	The preference by dark areas can be silenced by anxiolytic drugs.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
3. Catwalk (Deacon R.M.J., 2013)	The animal traverses a glass plate voluntarily and the footprints are recorded.	Locomotor activity and nociception.	Minimal physical interference and pain behaviors can be detected and analyzed (different patterns) by this method. Analgesic efficiency can be evaluated.	Non invasive // Mild discomfort	Locomotion

4. Cued and Contextual Fear Conditioning (Curzon P. et al., 2009)	The animal is placed in a box where different expected/unexpected stimulus (light, tone, odor) will be presented. The animal will freeze when an unexpected stimulus is presented. The percentage spent freezing and time for extinction will be measured.	Associative learning and memory (amygdala-hippocampus dependent).	Rodent models with cognitive deficits show less freezing during both cued and context freezing after having been conditioned. Furthermore, they show faster extinction of conditioning compared to WT animals.	Non invasive // Moderate discomfort	Behavior (cognition)
5. Electromyography (EMG) (Osuchowski M.F., et al., 2009)	Different electrodes are located in the paw and in the tail. The time required to transport the electric signal is recorded.	Skeletal muscle electric activity.	In MG rats, EMG is useful to record the degeneration of the muscles and the progression disease.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Neurologic
6. Elevated zero maze test (Kulkarni SK. et al., 2007)	Animals are displaced in an annular or "X" shape elevated platform with open and enclosed quadrants. The time spent in enclosed areas is measured.	Anxiety.	In rodent with cognitive deficits, spent less time in the enclosed arms compared to WT animals, indicating memory deficits.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
7. Encephalography (EEG) (Xie C. et al., 2011)	Brain spontaneous activity is measured and recorded from multiple electrodes placed on the scalp. A video recording is necessary to confirm epileptic seizures.	Electrical brain activity and epileptic seizures.	Epileptic events and encephalopathies can be diagnosed by this method in animal models.	Invasive // Mild discomfort (under anesthesia)	Neurologic
8. Forced swimming tests (Can A. et al., 2012)	The animal is placed in a cylindrical tank with water (the bottom is not reachable and they can not scape from the container). The time spent trying to escape/swim/float is recorded.	Anhedonia, depressive-like behavior	Anti-depressant therapies can be tested using this technique.	Non invasive // Moderate discomfort (anxiety and stress)	Behavior (affect)
9. Horizontal and fixed bars (Deacon RMJ. 2013)	The animal is placed in the middle of a horizontal suspended bar (with different diameters) where they need to walk without falling. In the fixed bars, the different diameters bars are fixed and they need to go from one end of the stick to the base.	Motor function, orientation and coordination	The animal get a composite score: time spent on the bar without falling, capacity to reach the base of the apparatus and the diameter of the bar. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort	Locomotion and neurologic
10. Intubation	Artificial ventilation is	Ensure breading	Electromyography is a	Invasive //	Operational

[redacted] et al., 2015)	provided to the animal through a trachea incision, placing a tube connecting to air flow.		procedure where curare is injected, blocking all muscles making artificial ventilation necessary.	Mild discomfort (under anesthesia, terminal experiment)	
11. Morris Water Maze (Vorhees CV. and Williams MT., 2008)	The rodent is placed in a swimming arena where a submerged platform is located in a strategic place. The time spent to learn will determine the spatial learning and later on the time spent to find the platform will be correlated with the reference memory (the arena is divided by 4 quadrants).	Spatial learning and memory hippocampus dependent. Swimming skills	Rodents with cognitive deficits will spend less time in the target quadrant, because they forgot where it was. The swimming speed can also be measured to determine the locomotion status.	Non invasive // Mild discomfort	Behavior (cognition) and locomotion
12. Nerve conductance speed (NCS) (Osuchowski M. et al., 2009; [redacted] et al., 2015)	Electrodes are placed in the leg and on the tail of the animal. The time required to receive the stimulus from the emitting and the receiving is recorded and correlated with the nervous and muscle status	Nerve damage and neuropathic measurement	Nerve damage can be measured using this technique for example for sciatic nerve impairment. Neuropathy can be tested also as a drug side effect using this technique	Invasive // Mild discomfort (under anesthesia)	Neurologic
13. Object recognition task (Leger M. et al., 2013)	Two similar objects are presented to the animal for the first time and the second time one of them is replaced by a new object. Object exploration duration and habituation times can be analyzed.	Cognition and recognition memory	Animals with cognition impairment will spend more time to recognize the constant and the new object.	Non invasive // Mild discomfort	Behavior (cognition)
14. Olfactory function /Smell threshold concentration test (Yang M. et al., 2009; Katzay A. et al., 2014)	Animals are exposed to increasing concentrations of menthol diluted in mineral oil and the minimal menthol concentration that the mouse responds to is recorded. A mouse response is considered positive when it displays a sharp aversive movement away from the source of smell.	Olfactory function and depression like-behavior	A depressive like behavior is related to decreased olfactory function, so animals with depression will need a stronger olfactory stimulus for positive reaction	Non invasive // Mild discomfort	Behavior (affect)
15. Open Field Test (Bailey KR. And Crawley JN., 2009)	The animal is placed in a square chamber, divided in areas. A recording system will determine the	Locomotion, emotional and anxiety.	Rodent models with cognitive deficits or treated with anxiolytic drugs will show	Non invasive // Mild discomfort (anxiety)	Behavior (affect) and locomotion

	preference of the animal between the periphery and the central areas. In parallel, distance and speed, rare behaviors, grooming, etc. can be evaluated.		reduced time spent in corners due to reduced anxiety.		
16. Parallel rod floor test (Kamens HM and Crabbe JC., 2007)	The animal is placed in a parallel stainless steel rod with a base plate behind. The animal paws are recoded and the number of times they slip through the parallel metal rods and touches the base plate.	Motor coordination (ataxia) and activity.	In rodent with cognitive deficits, locomotion deficits have been observed.	Non invasive // Mild discomfort	Locomotion
17. Perfusion ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is injected in the central venous system with a reagent (PBS or a fixative)/ The liquid is injected in the left atrium and a cut in the right ventricle drain the liquid.	Remove blood and/or preserve the tissue and organs.	Muscles in MG animal models have to be analyzed ab for this, fixed material is necessary because it help to maintain the integrity of the proteins. Brain is another important organ where sometimes the blood has to be removed or the tissue has to be fixed.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Operational
18. Pre-pulse inhibition (PPI) (Powel SB et al., 2009)	The animal receives a weak prestimulus o prepulse that results in the inhibition of the reaction of the animal after a subsequent strong startling stimulus or pulse (usually acoustic or light stimulus).	Anhedonia, depressive-like behavior	PPI has been used primarily in pharmacological animal models to screen putative antipsychotic medications. It is considered to be one of the most promising neurophysiological indexes for translational research in psychiatry (Hidetoshi T et al., 2011).	Non invasive // Mild discomfort	Behaviour (affect)
19. Rack grabbing test [redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and a 300g metal rack has to be grabbed repeatedly (5 times with a resting interval of 30s aprox.).	Strength, weakness and fatigability. Depressive-like behaviors.	MG animal models or other muscle impaired models can be graded using this method by the number of repetitions and the time they grab the rack.	Non invasive // Mild discomfort (anxiety)	Locomotion, Behavior (affect)
20. Resident-intruder tests	The animal is placed in a box, first with a sterile	Social interaction, stress and	The social and communication skills	Non invasive // Mild	Behavior (affect) and locomotion

(Koolhaas JM. et al., 2013)	female and later with a male from a non-aggressive strain. Social interaction and stereotypical aggressive behaviors are recorded and later analyzed by the frequency of these activities.	aggressiveness/violence.	and the locomotion can be measured in transgenic mice, where the animals presented more violence. Also the drug effect can be studied in terms of clarity and aggressiveness.	discomfort (anxiety)	
21. Rotarod (Deacon RMJ. 2013)	The animal is placed in a horizontal cylinder (delimitated on the sides). The cylinder rotate at different increasing speeds and the animal have to walk without falling.	Motor function and coordination	The animal get a composite score: ledge test, hind limb clasping, gait, kyphosis (locomotion) and time spent. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort (anxiety)	Locomotion and neurologic
22. Social interaction test (Kaidanovich-Bejilin O. et al., 2011; Sato A. et al., 2013)	The animal is placed in a 3 chamber cage. After habituation, the preference between an empty/known animal/unknown animal is measured. The activity is recorded and number and time spend in each department counted.	Natural sociability and preference novel sociability.	Many neuropsychiatric disorders are characterized by social behavior and recognition disruption. Other locomotion and cognitive parameters can be recorded.	Non invasive // Mild discomfort	Behavior and locomotion (affect)
23. Spatial Y-maze, X-maze and V-maze spontaneous alternation test (Lainiola M. et al., 2014)	The animal is placed in the connection of a 3 arm maze and the exploring capacity is measured by the number of arm entries/alternation. Different objects can be placed at the end of the arms.	Cognitive deficits, memory, anxiety	Transgenic animals or drug effect can be analyzed in the cognitive level using this method, measuring the willingness of rodents to explore new environments. Memory deficits can be measured also by the time spent to recognize the objects.	Non invasive // Mild discomfort (anxiety)	Behavior (affect/ cognition)
24. Sucrose preference/anhedonia test (Strekalova T. et al., 2004)	The rodent is free to choose between a water/sucrose solutions. The % of preference sucrose/liquid consumed is used as criteria (threshold 65%).	Anhedonia and depression-like behaviors	In chronic stress animal model, depression like behavior shows the acuteness of the model with a reduction of sucrose consumtion.	Non invasive // Mild discomfort	Behavior (affect)
25. Tail suspension test ([redacted]. et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and they need to climb on the top of the hand repeatedly (5 times	Strength, weakness, fatigability. Stress and depressive-	In MG rats, this is a parameter used to observe the acuteness of the disease.	Non invasive // Mild discomfort (anxiety)	Locomotion and behavior (affect)

	with a resting interval of 30s approx.).	like behaviors.	Animals exposed to tail suspension for longer periods daily develop chronic stress.		
26. Venipuncture	A needle is used to puncture an occluded vessel (saphenous vein or submandibular venous sinus). The blood start dripping and after collecting the required amount, you have to press the area to stop the bleeding.	Obtaining a blood sample.	Blood contains a lot of parameters that can be studied in order to monitor autoimmune state in animal disease models like MG, encephalitis, etc. Drug side effects can also be assessed by blood analysis.	Invasive // Mild discomfort	Operational
27. Von Frey and Hargraves (Krzyzanowska A. And Avedaño C., 2012)	The animal is subjected to different thermic and mechanic increasing intensity stimulus. The maximum intensity the animal can withstand without reaction is recorded as a threshold.	Pain susceptibility/ nociception.	Animal models can be tested by this method to assess the susceptibility to pain which reflect their own welfare. Could be an indirect measurement of therapy efficiency or to detect side effects. It also helps to determine the general health.	Non invasive // Moderate discomfort	Behavior (pain)
28. Weight measurement	Animals are placed in a balance and the weight is recorded.	General health.	Weight is a general measurement related with general health, disease progression (for example in MG) and drug side effects.	Non invasive // Mild discomfort	Operational

[redacted]

[redacted]

- [redacted]
- [redacted]
- [redacted] nerve conductance speed and electromyography

Besides the cognition, neurologic and locomotor assessment, some other operational task may be applied to the animal models like general observation (not consider a test/task, since the animals will be checked routinely), venipuncture and weight measurements during the experiment and other terminal tasks like intubation or perfusion at the end of the experiment, depending on the specific purpose of the study.

The experiments will be run for shorter periods than active immunization studies (Appendix 2). For example, the passive immunization in MG is terminated 48-72h after induction (Kusner LL, et al., 2015). For other antigens from the CNS such as for NMDAr encephalitis, animals will be sacrificed 5-46 days after immunization (Planaguma J. et al., 2014) or a neuromyelitis optica (NMO) animal model which is injected with antibodies anti-aquaporin-4, which it takes 14 days (Kinoshita M. et al., 2009). Longer experiments studying the chronic pathological role of the antibody will reach maximum 46 days ([redacted] et al., 2016 under revision).

All the tests/procedures applied to the animals are well established procedures under a standard protocol (Standardized operational protocol; SOP) which will reduce the bias of the experiment, since in these cases, interaction between the different tasks are already described and we will not make these specific set of tests for the experimental animals. In case it is necessary to include tasks that will affect each other because of the research question, the groups will be divided in subgroups which will follow different sets of tests. On the other hand, another measure to reduce the bias is the design of the tests performed in each experiment, which will be defined by the literature available currently before the performance of the experiments and also based on the expertise of people who has large experience in the behavior test. Under their advice, the tests will be chosen following a stress increasing scale throughout the experiment (in example, an open field will be always performed before a forced swimming test). It is important to mention that all observers (minimum of 2 independent researchers) will be blinded to different groups when performing the tasks.

For this appendix the go/ no-go criteria are the following:

1. Animal phenotype
    a) Robust and standard: analysis on the different behavior, neurologic and/or locomotion tasks will be performed as well as *in vitro* analysis defining the levels of different markers involved in autoimmunity like autoantibodies (specific and unspecific), inflammation cells and molecules e.g. plasma cells and cytokines. For standard animal models, the non-reproduction of the symptomatology and other markers is a direct no-go criteria, taking into account the variance between experiments. For novel animal models, if no differences equal or higher than 10% between the disease and the control group are found, a no-go decision will be followed. In this case, the experimental conditions of the model will be studied in detail and changes (e.g. species, gender, route of administration, frequency, dose, adjuvants, etc.) will be implemented to try to generate a robust and standard animal model. If no differences are found after modifying the strategy, we will report the results as a non-successful OR NON-VIABLE model for the specific antigen of interest.
    b) Severe: the symptomatology of the animal model will be observed and in case of severe discomfort and/or pain, because of the intervention procedures (e.g. immunization), analgesic will be applied and if even using this the animal keep suffering, a no-go decision will be followed and as explained in point a), some changes will be implemented.

2. Test battery
   After the analysis of the results of the battery tests, a go/no go decision will followed. In case an animal model shows differences in one of the domains and not in the other, the number of tasks assessing this parameter will be increased and the other test removed from the batch. Moreover, some tests included in Table 2 will be performed in case a strange behavior is observed during routine observation. It is important to mention that, the behavioral battery may change based on results generated from the first groups tested (based on a go/no-go decision after a first characterization in case of CNS active immunization models). For example:
   a) When the animal model is new, a characterization of the results in each test will provide an overview of the effect of each antigen specific immunization in the animal model, so a go decision will be followed in this case up to enough reliable data is available.
   b) In case of new animal models when a test has been shown (see previous point a)) not to detect differences between groups using a positive and a negative control, it will be discarded from the behavioral battery for this specific animal model (this indicates that the test for that particular model is not useful (no go decision)).
   c) When the animal model is well characterized and the results from the test are unexpected, showing no differences between the groups, a no-go decision will be followed.

For the postmortem study, CSF and blood will be collected for analysis of different molecule levels (e.g. total immunoglobulin levels and specific isotypes and antigen specific immunoglobulin or other factors affected) and cell populations will be studied as well to see how different immune system related cells and molecules can vary due to the disease. On the other hand, tissues will be collected to be analyzed. Immune related organs – thymus, spleen, lymph nodes, bone marrow –, brain, muscles and other organs which can be affected specifically by the disease developed – intestines, gut, sciatic nerve, liver, kidney, etc.-. For tissue collection, based on the research question to be answered, different treatments can be applied to the tissue, being necessary to include in some experiments perfusion with different reagents (glutaraldehyde, paraformaldehyde, etc.) of the animals to obtain fixed tissue (e.g. for MG, 2% glutaraldehyde perfused muscles are necessary to study the neuromuscular junction structure).
The techniques we are going to use during this study are: cell based assay (CBA), immunohistochemistry (IHQ), immunofluorescent (IF), radioimmunoassay (RIA), ELISA, Western blot/Dot blot, Flow cytometry, polymerase chain reaction (PCR), electron microscopy can be used and are already operational in our group, based on the literature.

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

The numbers of animals will be kept to a minimum by collaboration with people who have extensive experience in the generation of autoimmune animal models.
The research strategy will be based on the use of the most informative and descriptive test set for each disease model under the advice of experts in the field, own experience and the use of the literature. We will try to include as many tests as possible taking always into account the bias error and also we will avoid superfluous repetitions. As mentioned above, go/no-go decisions will be taken according to the pathology. If no differences are found using a test already reported to show significant differences, the experiment will be halted. In case there is no previous report about the test, the experiment will continue as a characterization (see above paragraph test battery).

We will use the effect size as a quantitative measurement of the strength of the phenotype of the animal model. Taking into account the next assumptions: a very large-huge effect size (d=1.20-1.40; Sawilowsky, S. Journal of Modern Applied Statistical Methods, 2009) and a power of 0.80 (Cohen J., Statistical Power Analysis for the Behavioral Sciences, 1988), with an α=0.05 for two-tiled and α=0.025 for one tailed, we will need 9-12 animals per group.

As parameters we will use, in active immunization animal models: IgG titers and weight measurements with some phenotype characteristics (in case of CNS, all the tests proposed in the 5 domains that we will perform in the first pilot) to see whether there are differences or not between the animal model and the controls.
In case of passive transfer animal models, weight measurements and the outcome of the tests performed

to cover the 5 domains will be taking into account.

B. The animals

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

For this experiment, rodents, including mice and rats will be used. Different species, strain, and genetic background (also WT) animals can be included. The specie (3), strain based on the genetic background, gender (2) and age range will be adjusted according to the literature to the most suitable. According to the induced nervous system autoimmune pathology, the onset severity and progression of the disease can vary and also the extension of the model.

It is intended to use wild type animals (with or without LPS/encephalitogenic T-cells injection to permeabilize the BBB), models with and without an impaired BBB (i.e. ApoE$^{-/-}$) for brain targets and human transgenic models (i.e. epsilon or gamma AChR subunits (AChRε, AChRγ)) for the protein of interest to mimic the human pathology.
Based on previous literature for NDMAr encephalitis model; wild type C57Bl/6 can be used to mimic memory deficits, and anhedonic and depressive-like behaviors but not locomoter or epileptic phenotypes (Planaguma L. et al., 2014).
We have already experience with the MG model using female Lewis rats aged 10-12 weeks, which is suitable to distinguish the clinical characteristics. C57Bl/6J mice are recommended to study regulatory functions (Kusner LL. et al., 2015).

In general, we will use adult animals for experiments. However, it is known that many factors from the mother can affect the fetus. Antibodies are an example of these molecules that can cross the placental membrane and affect the fetus, even when the mother does not have symptoms, since the antibodies can target only fetal antigens, such as AChRγ. This has been investigated in autism spectrum disorder, where the mothers from autistic children showed a specific band against fetal brain but not against adult brain, meaning that the autoantibodies transferred by the mothers can recognize the fetal proteins, affecting the child. However, these bands were not found in those mothers with normal children, showing the specificity (Braunschweig D. et al., 2008). This can be also the case of other neuropsychiatric disorders like schizophrenia or bipolar disorder, among others. Because of this, we would like to include pregnant animals to study placenta transfer of antibodies and effects of those antibodies on the offspring viability and on the fetus/neonate.

For the animal models of passive immunization we will generate a maximum of 8 animal models (defined by the time period of 5 years). An estimated distribution can be: 2 animal studies for MG (PTMG), 5 CNS autoantibody studies and 1 model generated with autoantibodies generated *in vivo* (Appendix 1) but since the source of the autoantibodies is mainly from patients, is completely dependent on this. For these studies we will need about 30 animals per group (passive transfer autoimmune models are more robust since autoantibodies are directly administrated to the animal) and the same amounts of control animals.
When studying the role of the BBB, there will be 4 study groups consisting of:

1. Disrupted BBB – pathogenic immunoglobulin
2. Disrupted BBB – nonpathogenic immunoglobulin
3. Non disrupted BBB (wild type) – pathogenic immunoglobulin
4. Non disrupted BBB (wild type) – nonpathogenic immunoglobulin

For the development of all animal models, using a maximum of 8 antigens, this will be a total maximum of 960 animals (30*4*8=960). Likely, we will be able to reduce these numbers by testing different autoantibody targets at the same time. For example, we expect to study AMPAr, GABABr and NMDAr (common outcome parameters) which could all be injected in parallel and thereby we would only need one control group for the 3 antigens. Also, some control group can be saved in case we want to compare the incidence of the BBB disrupted models in the pathology, since the non disrupted groups can be shared in this case.
In this calculation we have already taken into account that some animals will have the same conditions but will be sacrificed in different way according to the tissue analysis requirement and the high variability existent in passive immunization models.

Summarizing, a total of 960 animals (600 rats and 360 mice) will be included in the "Passive immunization in rodents" appendix.

All animals will be obtained from a registered supplier or a researcher who have specific animal models in case the registered supplier doesn't have it.

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.

Animal models of autoimmune diseases are required to study the *in vivo* pathogenic mechanisms, which will help to understand the procedures underlying the disease. Autoantibodies from a specific patient can be individually studied, increasing the personification of the pathogenic tracks in each patient and prove that the autoantibodies are the cause of the pathology, which is difficult to measure in lower organisms. Increasing the knowledge in this area will help the clinicians to apply more efficient treatments to the patients and to test these drugs, also *in vivo* experiments using the disease model are required. There are no other *in vitro* experimental approaches that can give us this information.
The number of animals will be keep to the minimum that can give statistical differences by combining different autoantibodies with similar outcomes and sharing the control groups as explained above. By doing power calculations for sample size and implementing go/no-go criteria we further limit the amount of animals used.
Refinement will take place by using anaesthesia and analgesia for the invasive procedures.

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

We are going to adapt the accommodation and the care to the need of the animals. To limit animal discomfort; anaesthesia and analgesia will be used to reduce the discomfort of the animals during the invasive procedures, like nerve conductance or for the terminal experiments like electromyography using curare where the animals have to be intubated (for more details see Table 1). Animals which are going to follow pain susceptibility test could not be under anesthesia (will not have anesthesia during a certain period before the test is performing (usually pain tests are run in the middle of the experiment, time enough after the immunization and before the terminal experiments, so the anesthesia is not interfere in any case). Because of this, if some animal present high discomfort, the subject will be withdrawn (in any case, this is not what we expect, since these specific tests are performed in chromic models and the humane endpoints are always took into account).

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

☒ 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.

Animals will be monitored daily. Anaesthetics and analgesics will be used to reduce the pain and discomfort of the animals due to some procedures (intra ventricular injection, NCS and EMG). Pain tests are going to be run during the experiment as a part of the model characterization or drug side effects and can be also used as a welfare measurment. No analgesics or anaesthetics will be used in normal procedures like blood extraction or neurological, motor or behaviour assesment/tests.

I. Other aspects compromising the welfare of the animals

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

All the animal models developed by passive immunization will develop a phenotype of moderate discomfort or in some cases will develop mild discomfort that will progress to moderate discomfort.
We observe and test the animals daily, which help us to describe the clinical status and the progression of the disorder. When animals reach humane endpoints, they are sacrificed to avoid extra suffering, which will be translated to a severe phenotype.

We can expect adverse effect of the immunization (infections in case of constant infusion through a pump, inflammation in the immunization site, immune system hyperactivity) and the induced disease *per se* due to the pathogenic mechanisms developed (the characteristics symptoms from each disease, i.e. weakness and fatigability in MG and stress, cognitive impairment and seizures in CNS models). Other aspects compromising the welfare of the animals is the *i.p.* injection, which increases the risk of peritonitis and serous fluid production.

There are no adverse effects are expected in relation to the venipuncture or the NCS, neither in the

behaviour nor in pain susceptibility tests.

Explain why these effects may emerge.

An unwanted effect could be observed because of immune reaction against the antigen and the permeabilizing agent. The animal will undergo inflammation in the area of injection with possible pain. Pain killers will be administered if necessary. Furthermore, systemic inflammation might occur if the antibodies start reacting with unspecific endogenous proteins. Many of these antigens have never been used in rodent models and we cannot know how symptoms differ from the human condition.

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

The severity of the induced pathology fluctuates since acute illness can be induced depending on the dose, frequency and duration of the immunization. [redacted]

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.

Humane endpoints will be applied in case of rapid weight loss (>20% within 3 days without any improvement), severe acute inflammation at the immunization point (untreatable by the definition of the veterinarian). The following endpoints are unlikely for completeness we include them since some phenotypes have never been tested before. These includes prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
In case of generalized unexpected severe adverse reaction the experiment will be halted.

Indicate the likely incidence.

The severity of the induced pathology fluctuates since acute illness can be induced depending on the dose, frequency and duration of the immunization. All animals will be sacrificed when the disease is more acute, "full blown" to study the pathological mechanisms in a severe situation, which are usually time short time periods. An example of MG passive model, where in an experiment silencing an associated protein to the neuromuscular junction by [redacted] et al., no animal had to be sacrificed before the end of the experiment (max. 72h after immunization). However, an example of the CNS is the work run by Planaguma and colleagues, where the animals had a constant i.v. infusion to reproduce a chronic psychiatric disorder, any of them reach the human endpoints before the end of the experiment (max. 42 days).
There is no literature available about the percentage of animals that will reach humane endpoints in the few experiments that have been performed. We predict a similar percentage of animals reaching humane endpoints, 0-5% as mentioned in appendix 2, since the targets are the same. However, it is important to mention, that animals will not build the immune response, since they will already receive the antibodies.
Because of all these evidences, we expect a reduced percentage of animals reaching the humane endpoints.

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').

All the animals will experience some degree of discomfort. The intermediate analysis of the behavioral, neurologic and/or locomotion tests cause mild to moderate discomfort. Operational procedures generate mild discomfort in 100% cases, however, immunization evokes moderate discomfort. Since all the animals will undergo immunization (except the control group for pain tests), the added discomfort of all tasks is moderate.

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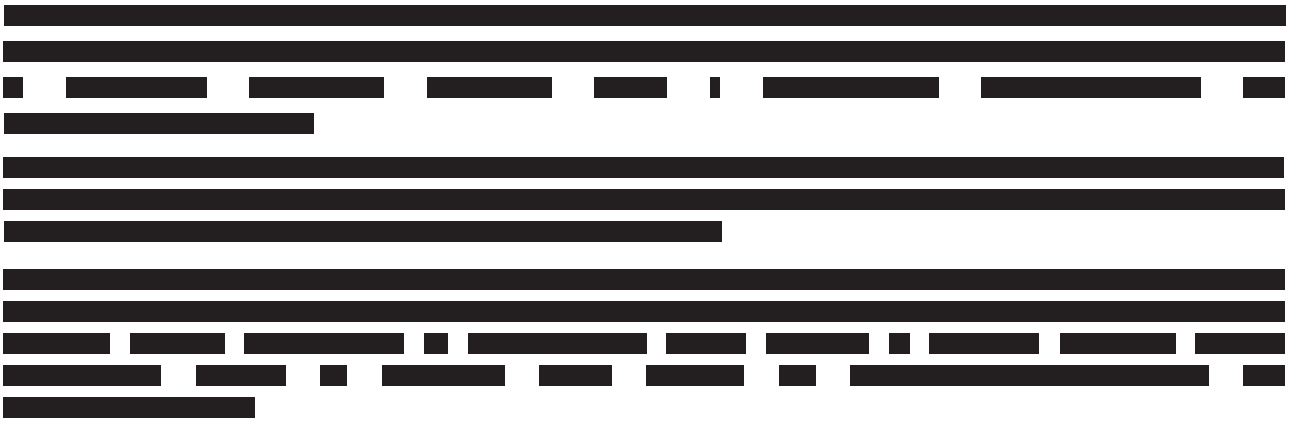

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End of experiment

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.

Animals will be sacrificed at the end of the experiment if no adverse events happen.
The sacrifice of the animals will be done according to the analysis requirements, in this case, based on the tissue characteristics. If fixed tissues have to be studied, perfusion is necessary to preserve the organ/tissue in an optimal way. Otherwise, normal sacrifice methods (cervical dislocation) will be used.

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

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'.	10700
1.2	Provide the name of the licenced establishment.	Maastricht University
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: 4 Type of animal procedure: Therapy efficacy and side effect in nervous system autoimmune 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.

This appendix describes the application of treatments in a prevention (starting before the disease appears, usually at the same time of the immunization) or therapeutic regimen. The diseases developed are based in nervous system autoimmune disorders, central and peripheral, developed in rodents as described in Appendix 2 and Appendix 3, by active and passive immunization.

The primary outcomes parameters are at two levels: *in vivo* and postmortem analysis.

1. The *in vivo* measures will give information on behavior neurologic and motor status of the animals. The battery of tests used will vary depending on the autoimmune disease model, but the characterization of all the models will be based in behavior (memory, anxiety, etc.), neurologic (electromyography (EMG), nerve conductance speed (NCS)) and locomotion (motor abilities, weakness, fatigue, etc.). This set of tests allows us to better describe the animal status (see brackets in list below).
2. The analysis of the material from the animal models obtained postmortem will be used to assess the biochemical and molecular level of the disease progression and explore the changes after treatment.

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

Therapies modulating the immune system are going to be tested in different nervous system autoimmune disease animal model described in Appendix 1 and 2. Chemical components with autoimmune pathways modulator effect [redacted] et al., 2010), antibodies targeting molecules of the

nervous system (Stevens et al., under preparation), cells playing a role in the mechanisms and genetic material (DNA or RNA) ([redacted] et al., 2009, [redacted] et al., 2016) changing the mechanism of some molecules, essential in the autoimmune diseases, are some examples of the possible therapies that can be applied in this project.
The administration via (s.c. and i.p. are the most common used and electroporation for genetic material using viral or non-viral vehicles), frequency, concentrations and amount of the drugs will be adjusted to the disease model (chronic or acute) and the drug used, based on the reported literature. In case no previous studies have been reported, different administration via and concentrations will be tested in a pilot study to define the optimal parameters (kinetic and chemodynamic properties of the drug will be taken into account) to have the most effective conditions. The treatment efficacy will be assessed to see whether there is a therapeutic window where the treatment is more effective, depending on the disease developed (acute or chronic) and the therapy agent administrated (e.g. in MG rat developed by active immunization, the therapeutic window goes from 4-8 weeks).

To evaluate the efficacy and the side effects of the drugs for the treatment, behavioral, neurological and locomotor tests will be performed in the animal model groups defined in advance and its controls (animals injected with vehicle like saline) (controls of the animal model are also always included to detect the effect of the drug on healthy animals). The outcomes of these tests in the different groups will allow us to assess whether a drug is effective in halting or improving the clinical symptoms of the pathology studied, and therefore provide a stronger translational potential then just analyzing the effect of the drugs using only outcomes from tissue. The animals will undergo the tests regularly (unless they can only be performed once –e.g. EMG) to have different time point data, where a progression of the pathology and drug effect can be studied. Usually, drug studies are performed in chronic disease models to see the progression of the disorder and the effect of the therapy over time and to characterize and had as much information as possible, regular tests are required, like blood samples ([redacted] et al., 2010).

Table 1. Procedures required characterizing neuropsychiatric animal models. Tests are classified according to the main outcome and the character of it: behavior, neurologic and locomotion. A brief description per test and the degree of invasiveness and discomfort has been included.

Procedure/test	**Description**	**Main outcome**	**Example**	**Invasive/ non invasive// discomfort**	**Behavior/ neurologic/ locomotion/ operational**
1. Alternating Y-maze (Hughes RN., 2004)	The animal is introduced in the center of a Y-shaped maze with three white opaque arms. The number of arm entries and the percent of alternations are calculated.	Willingness to explore new environments. Cognitive working memory.	Individuals with cognitive deficits perform less alternation compared to wild type (WT) animals due to the deficit in cognitive working memory.	Non invasive // Mild discomfort (anxiety)	Behavior (cognition)
2. Black and white test box (Costall B. et al., 1989)	The animal is introduced into a box with bright and dark areas. The preference light/dark areas are measured.	Anxiety and exploratory behavior.	The preference by dark areas can be silenced by anxiolytic drugs.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
3. Catwalk (Deacon R.M.J., 2013)	The animal traverses a glass plate voluntarily and the footprints are recorded.	Locomotor activity and nociception.	Minimal physical interference and pain behaviors can be detected and analyzed (different patterns) by this method. Analgesic efficiency can be evaluated.	Non invasive // Mild discomfort	Locomotion

4. Cued and Contextual Fear Conditioning (Curzon P. et al., 2009)	The animal is placed in a box where different expected/unexpected stimulus (light, tone, odor) will be presented. The animal will freeze when an unexpected stimulus is presented. The percentage spent freezing and time for extinction will be measured.	Associative learning and memory (amygdala-hippocampus dependent).	Rodent models with cognitive deficits show less freezing during both cued and context freezing after having been conditioned. Furthermore, they show faster extinction of conditioning compared to WT animals.	Non invasive // Moderate discomfort	Behavior (cognition)
5. Electromyography (EMG) (Osuchowski M.F., et al., 2009)	Different electrodes are located in the paw and in the tail. The time required to transport the electric signal is recorded.	Skeletal muscle electric activity.	In MG rats, EMG is useful to record the degeneration of the muscles and the progression disease.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Neurologic
6. Elevated zero maze test (Kulkarni SK. et al., 2007)	Animals are displaced in an annular or "X" shape elevated platform with open and enclosed quadrants. The time spent in enclosed areas is measured.	Anxiety.	In rodent with cognitive deficits, spent less time in the enclosed arms compared to WT animals, indicating memory deficits.	Non invasive // Mild discomfort (anxiety)	Behavior (affect)
7. Encephalography (EEG) (Xie C. et al., 2011)	Brain spontaneous activity is measured and recorded from multiple electrodes placed on the scalp. A video recording is necessary to confirm epileptic seizures.	Electrical brain activity and epileptic seizures.	Epileptic events and encephalopathies can be diagnosed by this method in animal models.	Invasive // Mild discomfort (under anesthesia)	Neurologic
8. Forced swimming tests (Can A. et al., 2012)	The animal is placed in a cylindrical tank with water (the bottom is not reachable and they can not scape from the container). The time spent trying to escape/swim/float is recorded.	Anhedonia, depressive-like behavior	Anti-depressant therapies can be tested using this technique.	Non invasive // Moderate discomfort (anxiety and stress)	Behavior (affect)
9. Horizontal and fixed bars (Deacon RMJ. 2013)	The animal is placed in the middle of a horizontal suspended bar (with different diameters) where they need to walk without falling. In the fixed bars, the different diameters bars are fixed and they need to go from one end of the stick to the base.	Motor function, orientation and coordination	The animal get a composite score: time spent on the bar without falling, capacity to reach the base of the apparatus and the diameter of the bar. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort	Locomotion and neurologic
10. Intubation	Artificial ventilation is	Ensure breading	Electromyography is a	Invasive //	Operational

([redacted] et al., 2015)	provided to the animal through a trachea incision, placing a tube connecting to air flow.		procedure where curare is injected, blocking all muscles making artificial ventilation necessary.	Mild discomfort (under anesthesia, terminal experiment)	
11. Morris Water Maze (Vorhees CV. and Williams MT., 2008)	The rodent is placed in a swimming arena where a submerged platform is located in a strategic place. The time spent to learn will determine the spatial learning and later on the time spent to find the platform will be correlated with the reference memory (the arena is divided by 4 quadrants).	Spatial learning and memory hippocampus dependent. Swimming skills	Rodents with cognitive deficits will spend less time in the target quadrant, because they forgot where it was. The swimming speed can also be measured to determine the locomotion status.	Non invasive // Mild discomfort	Behavior (cognition) and locomotion
12. Nerve conductance speed (NCS) (Osuchowski M. et al., 2009; [redacted] et al., 2015)	Electrodes are placed in the leg and on the tail of the animal. The time required to receive the stimulus from the emitting and the receiving is recorded and correlated with the nervous and muscle status	Nerve damage and neuropathic measurement	Nerve damage can be measured using this technique for example for sciatic nerve impairment. Neuropathy can be tested also as a drug side effect using this technique	Invasive // Mild discomfort (under anesthesia)	Neurologic
13. Object recognition task (Leger M. et al., 2013)	Two similar objects are presented to the animal for the first time and the second time one of them is replaced by a new object. Object exploration duration and habituation times can be analyzed.	Cognition and recognition memory	Animals with cognition impairment will spend more time to recognize the constant and the new object.	Non invasive // Mild discomfort	Behavior (cognition)
14. Olfactory function /Smell threshold concentration test (Yang M. et al., 2009; Katzay A. et al., 2014)	Animals are exposed to increasing concentrations of menthol diluted in mineral oil and the minimal menthol concentration that the mouse responds to is recorded. A mouse response is considered positive when it displays a sharp aversive movement away from the source of smell.	Olfactory function and depression like-behavior	A depressive like behavior is related to decreased olfactory function, so animals with depression will need a stronger olfactory stimulus for positive reaction	Non invasive // Mild discomfort	Behavior (affect)
15. Open Field Test (Bailey KR. And Crawley JN., 2009)	The animal is placed in a square chamber, divided in areas. A recording system will determine the	Locomotion, emotional and anxiety.	Rodent models with cognitive deficits or treated with anxiolytic drugs will show	Non invasive // Mild discomfort (anxiety)	Behavior (affect) and locomotion

	preference of the animal between the periphery and the central areas. In parallel, distance and speed, rare behaviors, grooming, etc. can be evaluated.		reduced time spent in corners due to reduced anxiety.		
16. Parallel rod floor test (Kamens HM and Crabbe JC., 2007)	The animal is placed in a parallel stainless steel rod with a base plate behind. The animal paws are recoded and the number of times they slip through the parallel metal rods and touches the base plate.	Motor coordination (ataxia) and activity.	In rodent with cognitive deficits, locomotion deficits have been observed.	Non invasive // Mild discomfort	Locomotion
17. Perfusion [redacted] et al., 2010; [redacted] et al., 2015)	The animal is injected in the central venous system with a reagent (PBS or a fixative)/ The liquid is injected in the left atrium and a cut in the right ventricle drain the liquid.	Remove blood and/or preserve the tissue and organs.	Muscles in MG animal models have to be analyzed ab for this, fixed material is necessary because it help to maintain the integrity of the proteins. Brain is another important organ where sometimes the blood has to be removed or the tissue has to be fixed.	Invasive // Mild discomfort (under anesthesia, terminal experiment)	Operational
18. Pre-pulse inhibition (PPI) (Powel SB et al., 2009)	The animal receives a weak prestimulus o prepulse that results in the inhibition of the reaction of the animal after a subsequent strong startling stimulus or pulse (usually acoustic or light stimulus).	Anhedonia, depressive-like behavior	PPI has been used primarily in pharmacological animal models to screen putative antipsychotic medications. It is considered to be one of the most promising neurophysiological indexes for translational research in psychiatry (Hidetoshi T et al., 2011).	Non invasive // Mild discomfort	Behaviour (affect)
19. Rack grabbing test ([redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and a 300g metal rack has to be grabbed repeatedly (5 times with a resting interval of 30s aprox.).	Strength, weakness and fatigability. Depressive-like behaviors.	MG animal models or other muscle impaired models can be graded using this method by the number of repetitions and the time they grab the rack.	Non invasive // Mild discomfort (anxiety)	Locomotion, Behavior (affect)
20. Resident-intruder tests	The animal is placed in a box, first with a sterile	Social interaction, stress and	The social and communication skills	Non invasive // Mild	Behavior (affect) and locomotion

(Koolhaas JM. et al., 2013)	female and later with a male from a non-aggressive strain. Social interaction and stereotypical aggressive behaviors are recorded and later analyzed by the frequency of these activities.	aggressiveness/violence.	and the locomotion can be measured in transgenic mice, where the animals presented more violence. Also the drug effect can be studied in terms of clarity and aggressiveness.	discomfort (anxiety)	
21. Rotarod (Deacon RMJ. 2013)	The animal is placed in a horizontal cylinder (delimitated on the sides). The cylinder rotate at different increasing speeds and the animal have to walk without falling.	Motor function and coordination	The animal get a composite score: ledge test, hind limb clasping, gait, kyphosis (locomotion) and time spent. Animals with neurological or locomotor issues will have difficulties and fall soon.	Non invasive // Mild discomfort (anxiety)	Locomotion and neurologic
22. Social interaction test (Kaidanovich-Bejilin O. et al., 2011; Sato A. et al., 2013)	The animal is placed in a 3 chamber cage. After habituation, the preference between an empty/known animal/unknown animal is measured. The activity is recorded and number and time spend in each department counted.	Natural sociability and preference novel sociability.	Many neuropsychiatric disorders are characterized by social behavior and recognition disruption. Other locomotion and cognitive parameters can be recorded.	Non invasive // Mild discomfort	Behavior and locomotion (affect)
23. Spatial Y-maze, X-maze and V-maze spontaneous alternation test (Lainiola M. et al., 2014)	The animal is placed in the connection of a 3 arm maze and the exploring capacity is measured by the number of arm entries/alternation. Different objects can be placed at the end of the arms.	Cognitive deficits, memory, anxiety	Transgenic animals or drug effect can be analyzed in the cognitive level using this method, measuring the willingness of rodents to explore new environments. Memory deficits can be measured also by the time spent to recognize the objects.	Non invasive // Mild discomfort (anxiety)	Behavior (affect/ cognition)
24. Sucrose preference/anhedonia test (Strekalova T. et al., 2004)	The rodent is free to choose between a water/sucrose solutions. The % of preference sucrose/liquid consumed is used as criteria (threshold 65%).	Anhedonia and depression-like behaviors	In chronic stress animal model, depression like behavior shows the acuteness of the model with a reduction of sucrose consumption.	Non invasive // Mild discomfort	Behavior (affect)
25. Tail suspension test [redacted] et al., 2010; [redacted] et al., 2015)	The animal is held by the tail and they need to climb on the top of the hand repeatedly (5 times	Strength, weakness, fatigability. Stress and depressive-	In MG rats, this is a parameter used to observe the acuteness of the disease.	Non invasive // Mild discomfort (anxiety)	Locomotion and behavior (affect)

	with a resting interval of 30s approx.).	like behaviors.	Animals exposed to tail suspension for longer periods daily develop chronic stress.		
26. Venipuncture	A needle is used to puncture an occluded vessel (saphenous vein or submandibular venous sinus). The blood start dripping and after collecting the required amount, you have to press the area to stop the bleeding.	Obtaining a blood sample.	Blood contains a lot of parameters that can be studied in order to monitor autoimmune state in animal disease models like MG, encephalitis, etc. Drug side effects can also be assessed by blood analysis.	Invasive // Mild discomfort	Operational
27. Von Frey and Hargraves (Krzyzanowska A. And Avedaño C., 2012)	The animal is subjected to different thermic and mechanic increasing intensity stimulus. The maximum intensity the animal can withstand without reaction is recorded as a threshold.	Pain susceptibility/ nociception.	Animal models can be tested by this method to assess the susceptibility to pain which reflect their own welfare. Could be an indirect measurement of therapy efficiency or to detect side effects. It also helps to determine the general health.	Non invasive // Moderate discomfort	Behavior (pain)
28. Weight measurement	Animals are placed in a balance and the weight is recorded.	General health.	Weight is a general measurement related with general health, disease progression (for example in MG) and drug side effects.	Non invasive // Mild discomfort	Operational

[redacted]

[redacted]

An example of [redacted]

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

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

All the tests/procedures applied to the animals are well established procedures under a standard protocol (Standardized operational protocol; SOP) which will reduce the bias of the experiment, since in these cases, interaction between the different tasks are already described and we will not make these specific set of tests for the experimental animals. In case it is necessary to include tasks that will affect each other because of the research question, the groups will be divided in subgroups which will follow different sets of tests. On the other hand, another measure to reduce the bias is the design of the tests performed in each experiment, which will be defined by the literature available currently before the performance of the experiments and also based on the expertise of people who has large experience in the behavior test. Under their advice, the tests will be chosen following a stress increasing scale throughout the experiment (in example, an open field will be always performed before a forced swimming test). It is important to mention that all observers (minimum of 2 independent researchers) will be blinded to different groups when performing the tasks.

For this appendix the go/ no-go criteria are the following:

a) Robust and standard: analysis on the different behavior, neurologic and/or locomotion tasks will be performed and *in vitro* analysis as well, definitive the levels of different markers involved in autoimmunity like autoantibodies (specific and unspecific), inflammation molecules and cytokines. For standard animal models, the non-reproducibility of the symptomatology and other markers is a direct no-go criteria, taking into account the variance between experiments. For novel animal models, if no statistically significant differences between the disease and the control group are found, a no-go decision will be followed.
b) Effectiveness: animals administered with the therapeutic drug has to show an improvement of, at least, 10% when compared with the controls. If this is not the case, a no go decision will be followed. After this decision, modifications in the administration route or the doses will be checked and applied if an improvement is available and check again this point.
c) Side effects: in case animals administered with the therapeutic drug suffer an increase of discomfort and/or pain compared to the controls (reaching humane endpoints, section J), a no go decision will be followed. After this decision, modifications in the administration route or the doses will be checked and applied if an improvement is available and check again this point.

For the postmortem study, CSF and blood will be collected for analysis of different molecule levels (e.g. total immunoglobulin levels and specific isotypes and antigen specific immunoglobulin or other factors affected) and cell populations will be studied as well to see how different immune system related cells and molecules can vary due to the disease and the therapy administered. On the other hand, tissues will be collected to be analyzed. Immune related organs – thymus, spleen, lymph nodes, bone marrow –, brain, muscles and other organs which can be affected specifically by the disease developed or the therapy applied – intestines, gut, sciatic nerve, liver, kidney, etc.-. For tissue collection, based on the research question to be answered, different treatments can be applied to the tissue, being necessary to include in some experiments perfusion with different reagents (glutaraldehyde, paraformaldehyde, etc.) of the animals to obtain fixed tissue (e.g. for MG, 2% glutaraldehyde perfused muscles are necessary to study the neuromuscular junction structure). If no perfusions are required, cervical dislocation or CO will be the standard methods to sacrifice the animals.
The techniques we are going to use during this study are: cell based assay (CBA), immunohistochemistry (IHQ), immunofluorescent (IF), radioimmunoassay (RIA), ELISA, Western blot/Dot blot, Flow cytometry, polymerase chain reaction (PCR), electron microscopy can be used and are already operational in our group, based on the literature.

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

For the proposed experiment we will make use of a 2-way ANOVA for analysis of behavioral outcomes. Factors for this ANOVA will be phenotype (disease, control), and treatment (saline, prevention, treatment). By making use of a 2-way ANOVA, we can ensure that the minimum number of animals used, will result in maximum power to determine the contribution of the factors involved.
For analysis of plasma and CSF levels of cells/molecules of interest we will make use of a mixed model analysis. The reason for this is that we can account for any missing values due to failure in assays or missing samples from animals. Fixed factors will be the same as those for the behavioral, neurological and locomotor outcomes, as a covariance structure we will choose compound symmetry.
A power analysis will be performed using the software G*Power, which allows to do power analysis for multi-way ANOVA (and also non -parametric criteria/tests: Friedman test for 3 more matched groups).

As mentioned above, go/no-go decisions will be taken according to the pathology. If no differences are found using a test already reported as a significant difference, the experiment will stop. In case there is no previous report about the test, the experiment will continue as a characterization.

B. The animals

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

For this experiment, rodents, including mice and rats, will be used. Different species, strain, and genetic background (also WT) animals can be included. The specie (3), strain based on the genetic background, gender (2) and age range will be adjusted according to the literature to the most suitable. According to the induced nervous system autoimmune pathology, the onset severity and progression of the disease can vary and also the extension of the model as described in Appendix 1 and Appendix 2.

Control groups are animals sham immunized or injected with an isotype control antibody and on one hand they are useful to detect the effect of the drug when there is no pathology and to subtract it effects, if present, from the effect on the disease groups taking into account the different setups of administration. On the other hand, there is another control group where the disease animals (and the controls), are injected with saline instead of the drug, which will give us the information of the correct development of the pathology (variances between experiments and animals have been observed) and that nothing is related with the injection procedure.

Using this experimental setup, both the effect of phenotype and the effect of the drug can be analyzed. Furthermore, it might be necessary to test different concentrations of the drug.

Based on theory, we estimate that we will need a maximum of 560 animals per drug study based on previous experience with similar models, for example to show a significance reduction of autoantibody titers and disease scores in an EAMG model treated with proteasome inhibition. The maximum experiment size will be 140 ((20 animal/study group*4 groups) + (12 animal/ control group*4 groups)=128 animals + 12 animal for the pain test blank = 140), with 3 replication experiments + 1 dose finding study (140*4=560 animals). In this calculation we are taking into account that some animals will have the same conditions but will be sacrificed in different way according to the tissue analysis requirement for every drug and/or the possible necessity to divide the groups in 2 subgroups because of the tasks overlapping. Since we are planning to test 5 possible drugs, this will bring the maximum amount of animals to 2800. However, we expect that the final group size will be significantly smaller after a power calculation. If possible, we will make use of historical controls or control groups generated in previous studies to lower animal numbers and interim analysis to reduce group size.

Summarizing, a total of 2800 animals (1680 rats and 1120 mice) will be included in the "Therapy efficacy and side effect in nervous system autoimmune models" appendix.

All animals will be obtained from a registered supplier or a researcher who have specific animal models in case the registered supplier doesn't have it.

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.

In terms of replacement, these drugs have been validated extensively by *in vitro* experiments to prove their mechanism of action. However, current *in vitro* systems are not able to model all the aspects of these nervous system autoimmune pathologies which are a complex multifactorial disease involving the contribution of many systemic components. While some of these drugs are already used in humans for the treatment of e.g. multiple myeloma, these drugs first have to show beneficial effects in animal models before they can be used in humans. While we expect that the drugs will be beneficial, there might be unforeseen effects which may result in a worsening of the diseases. Therefore, these drugs first have to be evaluated in an animal model.
With the following experiment we want to validate the use of these drugs in different experimental nervous system autoimmune diseases and if we observe a significant beneficial effect the approval for clinical trials will be faster.
In terms of reduction we can limit the amount of animals by investigating several parameters at once like the contribution of the different phenotypes (disrupted BBB, different autoimmune diseases, etc.). Furthermore, because we are planning on testing at least 4 different drugs, we will analyze if it is possible that historical controls can be used, if cohort effects are absent. This will further limit the number of animals. Next, we also choose an animal model with strong behavioral effects. Furthermore, we will perform intermediate analysis to further reduce the number of animals if possible. By doing power calculations for sample size and implementing go/no-go criteria we further limit the amount of animals used.
In terms of refinement, we have chosen to use the most suitable animal model for each pathology (Appendix 2 and 3). This will limit the amount of time that animals are in experiment, and thus experience possible discomfort. Finally, we are going to adapt the accommodation and the care to the

need of the animals.

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

We are going to adapt the accommodation and the care to the need of the animals. To limit animal discomfort; anaesthesia and analgesia will be used to reduce the discomfort of the animals during the invasive procedures (see Appendix 2 and Appendix 3). The interaction with the therapeutic agent tested has to be checked before. If the discomfort can be reduced, the subject will withdraw the experiment.

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

☒ 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.

The animals will be checked daily for any sign of pain or discomfort due to treatment or any other experimental procedures hereby described, and if any of these symptoms are detected, analgesics will be applied to relief the suffering.

I. Other aspects compromising the welfare of the animals

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

We can expect adverse effect of the immunization and of the induced disease *per se*, for example, EAMG cause weakness and fatigability and in case of CNS, memory and some neurological and locomotors impaired abilities can be expected (we can't be sure since no literature is available at the moment), which generate stress as explained more in detail in section K. There are no adverse effects related with the venipuncture or the NCS, neither in the behaviour or pain susceptibility tests.
Animals might experience adverse reaction because of the drugs administered and discomfort may be observed due to the pathology the model develops. These outcomes include increased anxiety, cognitive and neurological impairment locomotor deficits and stereotypical behavior. Furthermore, in some neuropsychiatric animal models, epileptic attacks may be observed. Other aspect compromising the welfare of the animals is the *i.p.* injection, which increases the risk of peritonitis and serous fluid production.

Explain why these effects may emerge.

Although some drugs are already used for other indications, some drugs have never been tested before. Therefore these drugs might cause unwanted side effects that may cause discomfort. Depending on dosage they are potent immunosuppressor. The dose that will be administered will be adjusted to the optimal conditions based on the literature or on dose finding pilot studies. However, unexpected interactions due to the autoimmune pathology cannot be ruled out.

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

Regular monitoring (daily) will prevent and minimize the severity of the welfare since the animals are going to receive pain killer, anesthetics and/or analgesics in case they are necessary (and allowed with the therapeutic agent). The administration site will be checked frequently (the immunization side as well as described in Appendix 2 and Appendix 3) to be sure that no complications appear. If any adverse reactions occur, drug dose will be lowered, or the experiment will be halted. Human endpoints are going to be considered if the animal showed a severe discomfort and euthanasia is going to be applied if the individual reach that status.

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.

Humane endpoints will be applied in case of rapid weight loss (>20% within 3 days without any improvement), severe acute inflammation at the immunization point (untreatable by the definition of the veterinarian). The following endpoints are unlikely for completeness we include them since some phenotypes have never been tested before. These includes prolonged diarrhea (>3 days), coughing, self-induced trauma, icterus and severe ulceration or bleeding (Recognition of pain and distress, Principles of Laboratory Animal Science, 2001).
We expect neurological symptoms related with autoimmune models where the CNS is target. The incapability to perform the behaviour tasks, the inhability to eat and drink (recognized by dehidratation sings, weight loss and general body condition) or the presence of continious tremors, status elipticus or circling (symptoms from what the animal will not recover) are non expected symptoms that, in case of occur, will be taken as a humane endpoint and the animal will be sacrified.
So human endpoints will be based on adverse reaction due to treatment. In case of unexpected severe adverse reaction the experiment will be halted.

Indicate the likely incidence.

The incidence will be dependent on the induced disease (Appendix 2 and 3), the character of it: severe or chronic, and on the therapeutic agent and its effects/side effects based also on the dose and the frequency and administration via. Because many combinations can be assessed, is quite hard to determine the incidence value. We estimate the incidence to be extremely low, in the order of 0-5% after a dose finding study. However, for the dose finding study, the incidence cannot be estimated due to the facts that the drug could be a new drug never tested before in vivo and many doses and injections via will be tried or never tested for an specific animal model, where the parameters will be fixed as well.

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').

All the animals will experience some degree of discomfort. The intermediate analysis of the behavioral, neurologic and/or locomotion tests cause mild to moderate discomfort. Operational procedures generate mild discomfort in 100% cases, however, immunization evokes moderate discomfort. Since all the animals will undergo immunization (except the control group for pain tests), the added discomfort of all tasks is moderate.

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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.

Animals will be sacrificed at the end of the experiment if no adverse events happen. If the animal present severe discomfort or is suffering extremely, according to the human endpoint, this individual will be sacrificed.

The sacrifice of the animals will be done according to the analysis requirements, in this case, based on the tissue characteristics. If fixed tissues have to be studied, perfusion is necessary to preserve the organ/tissue in an optimal way. Otherwise, normal sacrifice methods (cervical dislocation, CO inhalation) will be used.

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

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD107002017808
Bijlagen
1

Datum 17 maart 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted],

Op 2 januari 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Autoimmunity of the nervous system" met aanvraagnummer AVD107002017808. Wij hebben uw aanvraag beoordeeld.

Op 15 februari 2017 heeft u uw aanvraag aangevuld. U heeft de vragen van de CCD beantwoord, de eerste bijlage in drie aparte dierproeven inhoudelijk gesplitst en uw aanvraag daarop aangepast.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
De algemene voorwaarde(n) zijn opgenomen op grond van artikel 1d lid 4, artikel 10a1 lid 2, artikel 10 lid 2 en/of artikel 10a3 van de wet.
De voorwaarde met betrekking tot het inzetten van muizen van beide geslachten in dierproef 'Generation of antigenic and antibody sources (2. Antibody generation)' en dierproeven 3.4.4.3-3.4.4.6 (Generation of antigenic and antibody sources (3. Acetilcholine receptor extraction); Active immunization in rodents; Passive immunization in rodents; Therapy efficacy and side effect in nervous system autoimmune models) is gesteld om het aantal dieren in voorraad gedood te verlagen.

U kunt met uw project "Autoimmunity of the nervous system" starten. De vergunning wordt afgegeven van 17 maart 2017 tot en met 1 februari 2022. De looptijd van de vergunning wijkt af omdat de startdatum in de aanvraag in het verleden ligt.

Overige wettelijke bepalingen blijven van kracht.

Datum:
17 maart 2017
Aanvraagnummer:
AVD107002017808

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-UM gevoegd. Dit advies is opgesteld op 2 januari 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
In aanvulling op het DEC-advies stelt de CCD voorwaarden. De voorwaarden staan in de vergunning beschreven. Voor het overige nemen wij het advies van de DEC over, inclusief de daaraan ten grondslag liggende motivering. Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

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

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

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

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

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

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

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

Datum:
17 maart 2017
Aanvraagnummer:
AVD107002017808

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Universiteit Maastricht
Adres:	Postbus 616
Postcode en plaats:	6200 MD MAASTRICHT
Deelnemersnummer:	10700

deze projectvergunning voor het tijdvak 17 maart 2017 tot en met 1 februari 2022, voor het project "Autoimmunity of the nervous system" met aanvraagnummer AVD107002017808, volgens advies van Dierexperimentencommissie DEC-UM. Hierbij is afgeweken van het DEC-advies. Er worden aanvullende voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is Associate Professor.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 2 januari 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 15 februari 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 15 februari 2017;
c Advies van dierexperimentencommissie d.d. 2 januari 2017, ontvangen op 2 januari 2017.
d De aanvullingen op uw aanvraag, ontvangen op 15 februari 2017

Aanvraagnummer:
AVD107002017808

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Generation of antigenic and antibody sources (1. Brain tissue isolation)				Deze proef is onderdeel van appendix 1.
	Ratten (Rattus norvegicus) / volwassen en embryo's	780	100% Licht	Er worden 180 volwassen ratten en 600 embryo's/ pasgeboren pups gebruikt.
3.4.4.4 Active immunization in rodents				Appendix 2.
	Muizen (Mus musculus) /	204	100% Matig	
	Ratten (Rattus norvegicus) /	816	100% Matig	
3.4.4.5 Passive immunization in rodents				Appendix 3.
	Muizen (Mus musculus) /	360	100% Matig	
	Ratten (Rattus norvegicus) /	600	100% Matig	
3.4.4.6 Therapy efficacy and side effect in nervous system autoimmune models				Appendix 4.
	Muizen (Mus musculus) /	1.120	100% Matig	
	Ratten (Rattus norvegicus) /	1.680	100% Matig	

Aanvraagnummer:
AVD107002017808

3.4.4.2 Generation of antigenic and antibody sources (2. Antibody generation)				Deze proef is onderdeel van appendix 1.
	Muizen (Mus musculus) /	60	100% Matig	
	Konijnen (Oryctolagus cuniculus) /	90	100% Matig	
3.4.4.3 Generation of antigenic and antibody sources (3. Acetilcholine receptor extraction)				Deze proef is onderdeel van appendix 1.
	Ratten (Rattus norvegicus) /	18	100% Matig	

Voorwaarden

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

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

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

3) Voor de muizen die voor het monoclonaal antilichaam experiment (Generation of antigenic and antibody sources (2. Antibody generation)) worden gebruikt (60 muizen) worden beide geslachten in evenredige aantallen gebruikt.

4) De IVD ziet erop toe dat voor dierproeven 3.4.4.3-3.4.4.6 (Generation of antigenic and antibody

sources (3. Acetilcholine receptor extraction); Active immunization in rodents; Passive immunization in rodents; Therapy efficacy and side effect in nervous system autoimmune models) een goed onderbouwde keuze voor het geslacht gemaakt wordt, gebaseerd op de beschikbare literatuur en gestandaardiseerde richtlijnen voor elk model. Indien mogelijk dienen beide geslachten te worden ingezet.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD107002017808

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 eihd kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

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

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

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