	Inventaris Wob-verzoek W16-18S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2016489								
1	Aanvraagformulier				x		x		
2	Projectvoorstel				x		x	x	
3	Bijlage beschrijving dierproeven 1 en 2				x		x	x	
4	Niet technische samenvatting	x							
5	DEC-advies				x		x	x	
6	Ontvangstbevestiging				x		x		
7	Adviesnota CCD		x						x
8	Beschikking en vergunning				x		x		

23 MAART 2016

AVD 1030020164 89

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☑ Ja > *Vul uw deelnemernummer in*	10300
☐ Nee > *U kunt geen aanvraag doen*	

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

Naam instelling of organisatie	Stichting Katholieke Universiteit Nijmegen
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	4 1 0 5 5 6 2 9

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

Straat en huisnummer	Geert Grooteplein 10
Postbus	9101, t.a.v. [redacted]
Postcode en plaats	6500HB Nijmegen
IBAN	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer	UMC St Radboud

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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

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

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

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

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: 21-04-2016
	Einddatum: 21-04-2021
3.2 Wat is de titel van het project?	From belly to brain: the role of gut bacteria in brain and behaviour using mouse models.
3.3 Wat is de titel van de niet-technische samenvatting?	Van buik naar brein: de rol van darm-bacteriën in autisme en attentie-stoornissen.
3.4 Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC: RU DEC
	Postadres: Postbus 9101, 6500 HB Nijmegen [redacted]
	E-mailadres: [redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☑ Nieuwe aanvraag Projectvergunning	€ 1.187,00	Lege
☐ Wijziging	€	Lege

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

- ☐ Via een eenmalige incasso
- ☑ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ■ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☐ Melding Machtiging
- ☒ DEC advies en factuurinformatie

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	[redacted]
Functie	[redacted]
Plaats	Nijmegen
Datum	[redacted]
Handtekening	[redacted]

2.

Form
Project proposal

- This form should be used to write the project propsal of 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'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	From belly to brain: the role of gut bacteria in brain and behaviour using mouse models of autism and attention disorders.

2 Categories

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

[X] Basic Research
[X] Translational or applied research
[] Regulatory use or routine production
[] Research into environmental protection in the interest of human or animal health or welfare dier
[] Research aimed at preserving the species subjected to procedures
[] Higher education or training

[] Forensic enquiries

[] Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

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

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

The main role of the gastrointestinal (GI) tract is to digest food, which provides the body with nutrients required for survival and reproduction. The GI tract is also equipped to defend the body from antigens, bacteria, parasites and toxins. The mammalian gut is colonized by commensal bacteria that (together with fungi and micro-organisms) make up the gut "microbiota". Microbiota contribute to health as they convert food into micronutrients, outcompete pathological microorganisms and control immune factors such as cytokine levels. Colonization of the gut occurs early in life, after birth. Recent new technologies such as isolation of microbial genomes (i.e. *microbiome)* have allowed for a better understanding of the microbiome in health and disease (Martinez, 2014). The microbiome consists on average of more than 1.000 species (Qin *et al*. 2010) and 7.000 strains (Ley *et al*. 2006), from which the Firmicutes and Bacterioides are the two most prominent phyla (Eckburg *et al.* 2005; Lay *et al.* 2011; Diamant *et al.* 2011). Disruptions of the microbiome have been associated with disorders such as obesity (Ley *et al*. 2006; Turnbaugh *et al*. 2008), inflammatory bowel disease (Dicksved *et al.* 2008; Frank *et al.* 2007), metabolic syndrome (Zhang *et al.* 2010), cancer (Lupton 2004), Crohn's disease (Dicksved *et al.* 2008), ulcerative colitis (Frank *et al.* 2007) and irritable bowel syndrome (Carroll *et al.* 2011).

The GI tract is the largest surface area within the body to be exposed to the outside world, and changes in food composition or potential threats are rapidly signalled to the brain. The gut and brain communicate bidirectionally through the so-called gut-brain axis, which involves several pathways such as the autonomic and enteric nervous system, the immune system and the neuroendocrine system. This gut-brain axis has received a lot of attention lately and it was found that gut bacteria are an important 'hub' in these gut-brain communication pathways. Firstly via the immune system; microbiota shape the adaptive immune system and control immune factors such as cytokines that reach and act on brain tissue (Foster et al, 2015). Secondly, gut bacteria produce neurochemicals and hormones that interact with the enteric and central nervous system and the neuroendocrine system (e.g., somatostatin, acetylcholine, dopamine and norepinephrine), (Roshchina, 2010; Asano et al, 2012).

Due to its role in the gut-brain axis, recently the idea was proposed that microbiota may be an important modulator of behaviour and cognition (Cryan and Dinan, 2012). Indeed, changes to the rodent microbiome as a result of antibiotic-use, ingestion of probiotics (=bacteria) or faecal transplants were shown to affect emotional behaviour and brain biochemistry (Mayer 2011; Neufeld *et al*. 2011; Eckburg *et al.* 2005). In rats, metabolites produced by microbiota were shown to affect (social) exploration and reversal learning (MacFabe *et al*. 2011). Furthermore, since colonization occurs during early life, it has been hypothesized that the microbiome may play a role in developmental disorders such as autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), (Borre *et al.* 2014). This is supported by the finding that most ASD and ADHD patients suffer from digestive tract problems, which could indicate microbiome disruptions (Gonzalez *et al*. 2011). Also, in ASD patients GI-tract complaints are strongly correlated with the severity of behavioural symptoms (Buie *et al*, 2010; Adams *et al*, 2011) as the gut and dietary factors can increase or decrease behavioural symptoms in a subgroup of people with autism spectrum disorder (ASD) (MacFabe, 2015; Frye et al. 2015; Cryan et al. 2012). In his review of 2015 MacFabe writes that studies show that SCFA metabolism products of the gut microbiome remarkably effects the physiology of the patients' brains and behavior. Through the use of a translational animal model, in vitro and clinical studies is shown that SCFAs have unique properties, in particular with neuroplasticity, memory function, GPCRs, intestinal physiology, tissue barrier permeability, oxidative stress, mitochondrial function, carnitine metabolism and epigenetics, which are involved in ASD. Furthermore, there are many potential clinical scenarios for genetic-environmental interactions, which are consistent with increased exposure to or decreased metabolism of SCFAs in an individual at risk for ASD. Furthermore, in a study of autistic individuals with comorbid GI symptoms compared to typically developing controls with GI symptoms, pyrosequencing of ileal and cecal biopsies revealed a specific association of Sutterella species and the presence of anti-Sutterella serum antibodies in those with autism (Hsiao, 2014). Also, significantly increased levels of various bacterially modulated metabolites are detected in the urine of autistic children. This suggest a possible interaction between gut dysbiosis, intestinal permeability, and metabolic dysfunction in autism (Hsiao, 2014; Kelly et al. 2015). Moreover, this dysfunctional intestinal barrier could permit a microbiota driven pro-inflammatory state with implications for the brain (Kelly et al. 2015; Kiliaan et al. 1998), as an increase in gut permeability could precede mucosal inflammation to induce the inflammatory response. This way a feed-forward cycle between inflammatory responses and barrier dysfunction can be created in which the mucosal inflammation lowers the amount of mucosal 5-HT and other monoamines. Along with its precursors, it has been demonstrated to play a direct role in the regulation of intestinal permeability and also seem to be involved in regulating behaviour (Kelly, 2015). Overall, this and other increasing evidence indicates that changes in the composition of the commensal microbiome can alter complex behaviours that are included in ASD, like anxiety-like behaviour, emotional or depressive behaviour, and locomotor activity among others (Hsiao, 2014; Gonzalez et al. 2011; MacFabe et al

2011; Mayer 2011; Neufeld et al. 2011; Eckburg et al. 2005; Cryan and Dinan, 2012). Besides these changes in the microbiome, there also seem to be differences at the level of the brain in autism. Current neuroimaging and postmortem studies have provided evidence for disruptions in functional and structural connectivity in the brains of individuals with ASD (Vissers et al. 2012). More specifically, the literature in ASD is currently ascertaining differences in short vs. long range connectivity as being core to the emergence of the phenotype (Kana et al. 2014).
In Attention-deficit / hyperactivity disorder (ADHD) less research had been performed. It is known though that ADHD is a neurological disorder associated with alterations in dopamine neurotransmission and deficits in reward processing. The microbiome may contribute to ADHD aetiology through the gut-brain axis as Pelsser et al. found improved cognitive symptoms with an ADHD dietary intervention (Pelsser et al, 2011). More recently, researchers in Nijmegen investigated whether there are differences in the microbiome between ADHD diagnosed patients and controls (data under review from Aarts et al.). Also, the connection with the neural reward processing was examined. From the results it is apparent there is differences in relative amounts of different bacterial taxa between patients and controls. They found a nominal increase of the genus Bifidobacterium in ADHD patients. [redacted]

Given these observations, a recent scientific commentary concluded that the neuroscience-microbiome field may offer exciting new findings in terms of mechanisms for brain functioning and psychiatric disease, but also recognized that research into underlying pathways of gut-bacteria-brain-behaviour interactions is still in its infancy (Cryan and Dinan, 2015).

As mentioned above, one of the pathways involved in the gut-microbiome-brain interaction is the immune system. Microbiota are a key regulator of the innate and adaptive immune system. Researchers in psychiatry and neuroscience are increasingly recognizing a role for the immune system in behaviour, and immune factors were found to influence anxiety, exploration and even higher level cognitive functions such as learning and memory and decision making in rodents (Cushman *et al*, 2003; Sankar *et al*, 2012, Donegan *et al*, 2014, Yirmiya and Goshen, 2014). A potential tool for modifying the immune system in the context of the gut-brain axis, is through oral application of bacterial extracts that act as immunostimulants. Such extracts are currently used in the treatment of infectious diseases and allergies, amongst others. A well-validated immunostimulant, which is also used in humans, is the immunomodulatory bacterial extract [redacted] (Schaad, 2010).

[redacted]

In conclusion, findings in both rodents and humans support a role for the microbiome in gut-brain communication. There is evidence that the microbiome can influence behaviour and cognition and that it is potentially involved in psychiatric disorders such as ASD and ADHD. Molecular pathways that subserve gut-bacteria-brain communication include the immune system and release of neurochemicals and hormones. Understanding such mechanisms would greatly enhance our understanding of the brain and has great therapeutic potential for brain disease, through dietary intervention. Therefore, the objectives of the research proposed here are 1. To study whether and how microbiome composition is affected in mouse models of ASD and ADHD

Finally, results of the proposed research will answer important biomedical research questions regarding the role of the microbiome in brain and be haviour. Including the discovery of biomarkers, as there is a need for surrogate outcome measures that are accessible (e.g. ratio of bacterial composition in the gut predicting behavioural traits) plus as a basis for target discovery (to ascertain if alterations in the gut microbiome could be viable therapeutic approach to ASD and ADHD).

For these mentioned reasons, the current project is expected to contribute to basic research as mentioned in section 2.1. But in addition to making a contribution to basic research, the proposed project is also expected to contribute to translational and applied research, as it is part of a translational study that has received funding from the 'NWO food and cognition' program. The experiments proposed in mice will be paralleled by a similar study in ASD and ADHD patients measuring microbiome differences, brain function (MRI) and behaviour. Therefore, these animal experiments will allow for translation between human and mouse findings and back. The data from mice will be essential by allowing study of brain tissue postmortem, in order to unveil the neurobiological underpinnings of the gut-bacteria-brain interaction.

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 main aim of this proposal is to better understand the relationship between the gut microbiome and brain and behaviour in mice, in the context of neurodevelopmental disorders. We plan to achieve this by addressing the following specific objectives:
(i) Objective 1. To study whether microbiome composition is affected in mouse models of ASD and ADHD, and to measure whether such differences correlate with the behavioural phenotype and measures of brain structure, neurotransmitter levels (both measured through MRI/ MRS) and molecular pathways (measured postmortem).
We ***hypothesize*** that in mouse models for ASD and ADHD the microbiome composition is different, when compared to healthy control animals. Additionally, we expect that such differences will correlate with behavioural and cognitive performance, and that this is reflected by changes in brain structure, neurotransmitter metabolites and molecular pathways.

(ii) Objective 2. [redacted]

The main objective of this project: *To better understand the relationship between the gut microbiome and brain and behaviour in the context of neurodevelopmental disorders*, is achievable within the proposed duration and with the available research infrastructure of our research group and institute for the following reasons:
- Firstly, results from Objective 1 will reveal whether or not ASD and ADHD mouse models differ in terms of gut microbiome composition. Results form this study (both positive and negative outcomes) will provide crucial information on whether microbiota are involved in the gut-brain axis in the context of neurodevelopmental disorders. The researchers involved in this project have experience with analyzing microbiomes, both directly and via an international network of EU-consortia [redacted]
- Secondly, within Objective 1 an 2 several parameters at the level of brain and behaviour will be assessed. The details of these read-outs will be further discussed in 3.4 and the table 'Description Animal Procedures'. In short, these techniques involve behavioural analysis using touchscreen-equipped operant chambers, in vivo MRI scanning of structural parameters using Diffusion Tensor Imaging (DTI) and Magnetic Resonance Imaging (MRS) and finally, postmortem molecular analysis of blood and brain tissue. Our lab (and department) have experience and a track record in all of these techniques, as reflected by publications using the touchscreen operant chamber [redacted] MRI/MRS data collection [redacted] and molecular analysis [redacted]

3.3 Relevance

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

Neurodevelopmental disorders such as ASD and ADHD have a major impact on the lives of patients and their families, as well as on society. ASD has a prevalence of about 1.5-2% and ADHD occurs in 5% of the adult population. Patients of ASD all face social, communication and language problems in a mild to severe degree. They also show restricted and repetitive patterns of behaviour, interests, or activities which can impair their daily

functioning. For ADHD problems as inattention, hyperactivity and impulsivity are present that will lead to social problems. Not listening to other people, being able to sit still or being able to wait their turn are just a couple examples of those problems arising in ADHD patients. For both disorders it is found that patients do not always respond well to treatment and the current pharmacological treatment options often come with side effects. Therefore, research which specifically targets novel pathways of the etiology of neurodevelopmental disorders is greatly needed, as such research may provide us with new biological insights and thus treatment options.

This project is expected to result in a better understanding of the mechanisms linking the microbiome to the brain via neural, endocrine and immune pathways in the context of ASD and ADHD. Up to date, validated biomarkers for the diagnosis and prediction of these disorders are lacking. This means that biomarker discovery is needed for surrogate outcome measures that are accessible (e.g. ratio of bacterial composition in the gut predicting behavioural traits) plus as a basis for target discovery (to ascertain if alterations in the gut microbiome could be viable therapeutic approach to ASD and ADHD). In addition, the technological advancements of the experiments proposed in this application (i.e. touchscreen operant boxes, MRI scanning) combined with microbiome research has not been reported before and allows the translation to brain networks involved in psychiatry (such as ASD and ADHD) to match with planned experiments in clinical populations. We will examine microRNA changes in different brain regions and serum samples from these animals post-mortem. Since microRNAs regulate gene expression, a change in the expression of these small RNA molecules can, in theory, be an important way by which putative functional phenomena can be explained. This novel hypothesis can therefore greatly improve our insight into the effect of microbiome on neural, endocrine and immune pathways, and can offer an explanation why there are only small genetic changes identified to be altered in patients. Besides that, behavioural models matched between human and animals, together will allow for a high degree of cross-species translation and rapid exchange of new findings. Taken together, this project might result in novel knowledge that might contribute to a better understanding of ASD and ADHD. So, it will pave the way to prioritize greatly needed biomarkers for ADHD/ASD and various other brain disorders and lead to further developing and testing new food/diet based therapeutic strategies.

3.4 Research Strategy

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

The research strategy of the proposed project aims to address the two objectives formulated earlier: 1. To investigate to which extend the microbiome is changed in mouse models of neurodevelopmental disorders ASD and ADHD and [redacted]

For this, the research proposed will be divided in two parts:
1. An initial study in which we plan to investigate whether the microbiome in animal models for ASD and ADHD is different to control groups [redacted] optimal dose and animal model. This second part is performed to further address the second objective by measuring high-level cognitive behaviours that require a long training duration, but have the particular benefit that these tests can reveal behavioural changes relevant in the context of ASD and/or ADHD.

Our strategy to understand microbiome differences in ASD and ADHD is that we have selected different mouse models for these disorders which all have a different origin in terms of disease etiology and that they exhibit different symptomatology. The mouse models of ASD and ADHD planned to

use in this research are the maternal valproate (VPA) mouse model for ASD which involved prenatal disturbance of neuronal development, the Dopamine Transporter knock-out (DAT KO) mouse model for ADHD which focusses on the dopamine system, and the Balb-cJ mouse model which has a genetic component. Another, reason for choosing the BALB/cJ model and their controls (BALB/cByJ) is that these are models that can model both features for ASD and ADHD [redacted] In humans the disorders are often comorbidly present, meaning that these patients have both ASD or ADHD. This combined phenotype is the basis for selecting the BALB/c model which demonstrates aspects of ADHD and ASD traits. The rationale for the DAT-/- (KO) mouse is that it is reported as a 'gold-standard' ADHD model and as such alterations in the microbiome in this model would be convincing to the field. Equally, the valproate (VPA) induced model (VPA) is seen as a 'gold-standard' model for ASD alone.

Valproate (VPA) mouse model:
Maternal valproate (VPA) exposure has permanent adverse effects upon neurological and behavioral development. C57/Bl6 mouse pups exposed in utero to VPA demonstrate difficulties in social interaction, altered ultrasonic vocalization communication, reversal learning as well as the differential expression of plasticity-related genes (also altered in human ASD)(Rinaldi et al. 2007; Roullet et al. 2010). In the current study, C57/Bl6 mouse pups at 8 weeks will be studied (following prenatal exposure to VPA in utero).

DAT-/- mouse model: The dopamine transporter knock-out mouse model is the specific model for ADHD, that do not effectively clear extracellular DA and exhibit a generally overactive dopaminergic state. The model demonstrate strong face, construct and predictive validity for ADHD, and spontaneous hyperactivity and impaired learning and memory. Recent studies have also demonstrated an impaired cliff-avoidance reaction in DAT-KO mice, indicating increased impulsivity. Unlike wild-type or heterozygous mice, DAT-/- mice are significantly more active in novel and home-cage environments and demonstrates learning and memory deficits in the 8-arm maze, novel object recognition and social food preference transmission tests (Yamashita et al. 2013; Gainetdinov et al. 1999; Wong et al. 2012). In the current study we will employ 8 week old DAT-/- (on a C57/Bl6 background strain).

BALB/cJ mouse model:
Finally, the Balb-cJ mouse model will be compared to the Balb-cByJ, these two substrains differ only with respect to a few copy number variations, but have a markedly different behavioural phenotype. The Balb-cJ (when compared to Balb-cByJ) was found to not only shows relatively low levels of social interaction in various settings and across various stages of development, but also other phenotypes with possible relevance to autism and ADHD, including relatively high levels of anxiety and aggressive behaviours, large brain size, underdevelopment of the corpus callosum, impulsive choices, hyperactivity and low levels of brain serotonin [redacted] Brodkin et al. 2007; Jacome et al. 2011; Fairless et al. 2008 & 2012; Bielas I. 2014; Mazei-Robinson and Blakely 2006; Leo 2013; Otobe and Makine 2004).

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.

As mentioned in the previous section (research strategy), the experiments will be conducted in two parts:

1. In the first study we plan to study the microbiome in animal models for ASD and ADHD and their controls, by means of faecal sample collection under baseline conditions in order to meet the first objective. This will be followed (in the same animals) by faecal sample collection during a 30-day treatment period [redacted] Oral gavage is necessary to control the exact intake amount. [redacted] At the end of the treatment animals will be euthanized and sacrificed to assess the blood, intestine and brain for changes. The details of this will be specified in the first 'Description Animal Procedures' table.

2. The second part of the research will make use of the results obtained in the first study, in which the optimal dosage and treatment duration were determined according to effects of treatment on immune markers in blood and intestine, and changes in brain and simple behaviours. First it should be mentioned that if one of the animal models does not show an effect on behaviour, that this model will not be further used in this second part of the project. Also, when the behaviour is different without any microbiome changes are observed, the second experiment will not be performed with that model. This second part is performed to further address the second objective by measuring high-level cognitive behaviours that require a long training duration, but have the particular benefit that these tests can reveal behavioural changes relevant in the context of ASD and/or ADHD. These high-level cognitive tests will be performed in a touchscreen-based operant setup. The applied tasks are analogue to a cognitive task battery used in human patients (the CANTAB battery) and can asses changes in behaviour across different cognitive domains. In the rodent analogues we will test the ASD and ADHD models on translational tasks of attention, impulsivity and cognitive flexibility (Mar et al. 2013; Oomen et al. 2013; Horner et al. 2013). Furthermore, this second study will be used to couple such behavioural findings to (more costly) measures of brain structural and functional connectivity using the 11.7-tesla MRI scanner in our research facility to be able to provide information on possible structural (MRI-DTI) and functional (resting state MRI) connectivity changes [redacted] The detailed timeline of this study will be specified in the second 'Description Animal Procedures' table.

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

This study assesses the influence of the microbiome on brain structure and function, cognition and behavior. In the pilot study the intervention of a bacterial extract [redacted] will be combined with two simple battery tasks while microbiome is collected at several time points. This way, the effect of the extract on the microbiome can be assigned to the behaviour seen during the performance of the task. The operant chamber tasks using touchscreen will, in the same way, subject the mice to behavioural tasks, where collected faeces are used to link the microbiome to the behaviour of the animals. [redacted] Finally, the microbiome collected can be correlated to possible changes in structure and function of the brain that are measured during the MRI measurement series at the end of the experiments. Overal, the experiments performed in the animals can provide more information on the relation between the microbiome and the brain, which is necessary to be able to translate this to human work that will be performed later by the same researcher.

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	Behaviour Pilot microbiome intervention
2	Behaviour in ASD and ADHD models with [redacted]

3.

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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 1	Type of animal procedure Behaviour Pilot microbiome intervention

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The aim of the experiments is to evaluate the effect of the microbiome on behaviour and cognition by examining the effect of dietary intake of an immunological bacterial extract that modulates immune/inflammation gut responses in mouse models of ASD and ADHD. For this purpose, we plan to study the microbiome in animal models for ASD and ADHD and their controls, by means of faecal sample collection of the mice, under baseline conditions and subsequently treating the animals during 10, 20 or 30-days with an immunological bacterial extract, called [redacted] This is done to assess optimal dosis and treatment duration. In addition, during the treatment, behaviour will be assessed by the use of two simple battery tasks and at the end of the treatment animals will be euthanized and sacrificed to assess the blood and brain for changes.

The approach to assess optimal dosis and treatment duration includes the following experiment:

[redacted]

To determine the dose and optimal duration of the treatment that result in the highest changes in the different measurements mentioned we will use the following primary end points that have successfully been used in previous studies performed by our research group and by other international research groups [redacted] :

- [redacted] (confidential, unpublished data). Those two dosages will be applied through oral gavage, which is necessary to control the exact intake amount, to separate experimental groups. In addition, other mice of the same strain will be part of control groups that receive vehicle or no treatment.
- Treatment will be performed in three blocks of 10 days, each followed by a 5-day break, chosen due to the duration of the treatment which has been used previously in mice, and to be sure the duration will have a translational value to humans. The measurement on day 3 after the last dose of the bacterial extract, makes sure that all the possible changes have taken place in the microbiome and to reduce the stress of the longitudinal administration.
- The effects of [redacted] treatment for different time points (10, 20 and 30 days) will be determined by assessing changes in multiple parameters:

1. Assessing simple behaviours:

In this set up the animals will perform two simple battery tasks to assess symptomatic behaviour of ASD and ADHD in the different strains. Both tasks are easy to perform and it does not require long training time. The first task performed will be the Y-maze Spontaneous Alternation task. This is a behavioural test for measuring compulsivity via the willingness of rodents to explore new environments. Over the course of multiple arm entries,

the subject should show a tendency to enter a less recently visited arm. The number of arm entries and the number of triads are recorded in order to calculate the percentage of alternation. The second test will be the Open Field. The Open Field task is a simple sensorimotor test used to determine outcomes as: general activity levels, gross locomotor activity, and exploration habits in rodent models of CNS disorders. These parameters will be assessed by analyzing recordings using an automated tracking system that is able to report: time spend in centre and pre-defined zones, overall mobility, distance moved and velocity within the first 5 minutes.

2. Blood and brains: In this set up a subset of the animals after each of the 10-day blocks will be euthanized at a certain time point after the behavioural measurements. The brains of the animals will be dissected and blood will be collected. The collected brains will be used for analysis at the transcriptional level. We will analyze the mRNA levels of different transcripts by RNA sequencing and/or qPCR of candidate genes in brain regions associated with the behavioural phenotype (e.g. ACC/vmPFC/OFC/striatum). Candidate genes will be selected on the basis of genetics landscapes of ASD and ADHD as published by our group (Poelmans et al., 2011; Poelmans et al., 2013). The blood will be used for analyzing hormone and lipid levels and immunological parameters. Steroids are powerful signalling molecules regulating a variety of physiological processes such as cell proliferation, cell differentiation, and reproduction. Variations in steroid concentrations may hint at hormonal imbalances that might play a role in diseases such as ASD and ADHD. Therefore the blood hormone levels will be analyzed by using ELISA assays. [redacted] Immunological assessment in the blood will be the final measurement. Candidate immunological markers chosen are specific for immune changes seen in ASD and ADHD [redacted]

3. Microbiome: The microbiome compositions from baseline and after a certain amount of doses of the treatment (10, 20 or 30) are analyzed from the collected droppings, as there is a need for surrogate outcome measures that are accessible (e.g. ratio of bacterial composition in the gut predicting behavioural traits) plus as a basis for target discovery (to ascertain if alterations in the gut microbiome could be viable therapeutic approach to ASD and ADHD). This will be done by using a validated method from [redacted]. From the droppings, we will examine primarily bacteria that alter monoamine signaling as the monoamines (dopamine, noradrenaline and serotonin) influence impulsivity-compulsivity and attention. However, we will also seek to conduct broader sequencing (Illumina-based 16S ribosomal RNA sequencing assays) which will be conducted at [redacted]. By using the 16S ribosomal RNA sequencing there will be a profile of phylogenetic marker genes to show the bacterial variability of the microbiome in the different strains and between the different treatment groups. This will seek to discover if there are other bacterial clusters that are related to these phenotypes [redacted], which can be used for future therapeutic approaches.

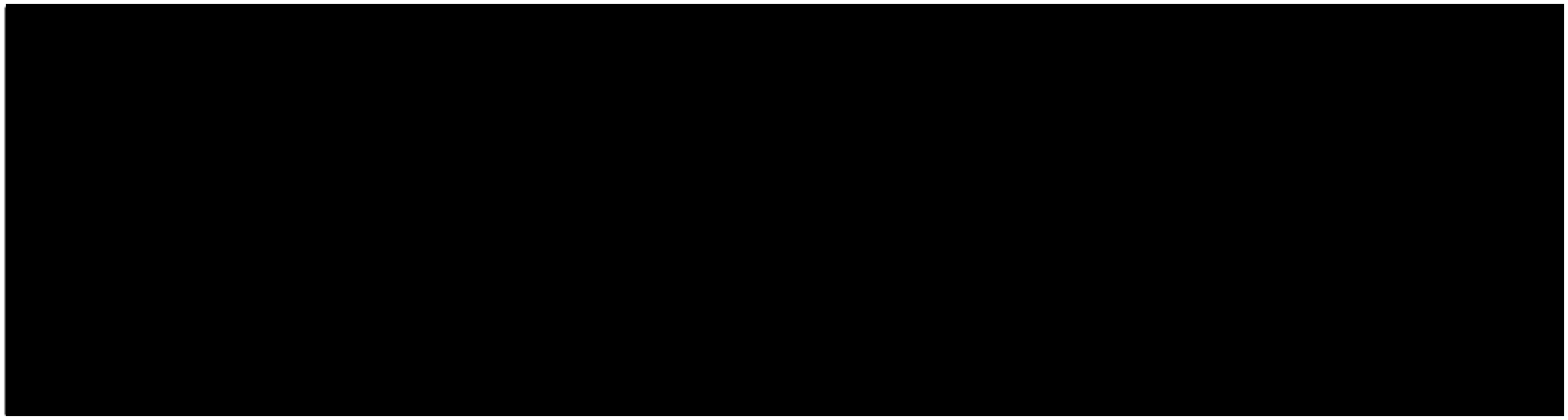
Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Pilot to determine optimal dose and treatment duration

A pilot study will be performed on four treatment groups per strain (BALB/cJ, VPA or DAT KO and matching controls). During the pilot study the animals will receive the [redacted] bacterial extract [redacted] to be able to set an optimal dose and treatment duration for the follow-up experiment, in which higher cognitive measurements are performed.

[redacted]

During the pilot we will start by collecting microbiome at baseline level (see **figure 1**). This will be done by collecting faeces of each animal using a validated work protocol received from [redacted] After that the animals are randomly assigned to a treatment group (**table 1**), as well as to a duration group. This means there are 12 different groups (treatments x duration) per strain.



The first treatment duration will be 10 days. Three of the groups will receive, The fourth group will not get any treatment nor oral gavage and will be the negative control for both conditions (table 1). After that an the animals will perform two simple battery tasks: Y-maze and the Open field. After that, a second sample will be collected of the microbiome by collecting the faeces for each animal again. After the collection of the behavioural data, this group of animals will be euthanized.
The second treatment duration will be 20 days. Again, three of the groups will receive, The fourth group will not get any treatment nor oral gavage and will be the negative control for both conditions (table 1). Just like the first group, an this phase the animals will perform two simple battery tasks: Y-maze and the Open field. After that, a second sample will be collected of the microbiome by collecting the faeces for each animal again. The phase will be followed by a second treatment block of 10 days. Then again a phase starts. The two simple battery tasks are performed again by the animals with subtle changes on set up for exploratory motivation, while keeping measurements comparable to the first behaviour. After the collection of the behavioural data, this group of animals will be euthanized.
The final, third treatment duration will be 30 days. As in the durations above, three of the groups will receive, The fourth group will not get any treatment nor oral gavage and will be the negative control for both conditions (table 1). In total these animals will get 30 days of treatment, divided in 3 blocks of 10 days. the animals will perform two simple battery tasks: the Y-maze and the open field. This means that the animals will perform this task two times, both moderately different (in terms of maze shape and testing room respectively) to ensure motivation to explore, but

keep measurements comparable. [redacted] After the collection of the behavioural data at day 43, this group of animals will be euthanized as well.

Food restriction

Before the start of the pilot, the weight of the animals will be slowly reduced to a percentage of 85-95% of the measured free-feeding weight by controlling the daily amount of food they are given (~2–5 g food per 25–35 g of body weight). This way the animals are subjected to a mild food restriction before the onset of the first treatment block, and continuing throughout the course of the experiment. Food restriction is implemented to mimic the conditions used during follow up experiments (ASD and ADHD) in which animals undergo cognitive testing in the touchscreen operant chambers and need to be restricted for motivational reasons.

Oral gavage training

Before starting with the pilot experiment, multiple researchers are trained to perform the oral gavage. This will be done to prevent damage to the oesophagus and to make sure there is always trained personal around during the performance of the experiments.

In more detail, the following experimental elements are carried out:

1. Assessing simple behaviours:

In this set up the animals will perform two simple battery tasks to assess symptomatic behaviour of ASD and ADHD in the different strains. Both tasks are easy to perform and it does not require long training time. The first task performed will be the Y-maze Spontaneous Alternation task and the second one is the Open field task.

Y-maze

This is a behavioural test for measuring compulsivity via the willingness of rodents to explore new environments as rodents tend to alternate arm visits and naturally avoid repeated entries ('spontaneous alternation'). This is reflecting natural exploration behaviour of a novel environment. In order to determine spontaneous alternation, mice will be placed in the long arm of a Y-maze and are allowed to explore the maze for 5 minutes. Over the course of multiple arm entries, the subject should show a tendency to enter a less recently visited arm. The number of unique arm entries and the number of triads are recorded in order to determine the percentage of spontaneous alternation behaviour.

Spontaneous alternation in the Y-maze will be assessed according to e.g. the Nature Protocols paper on spontaneous alternation in the T-maze by Deacon and Rawlins (2006).

Open field

The open field test (OFT) is a common simple measure of sensorimotor performance. It is used to determine outcomes as: general activity levels, gross locomotor activity, and exploration habits in rodent models of CNS disorders (Gould *et al.* 2009). Both the quality and quantity of the activity can be measured. Principally, the open field (OF) is an enclosure, generally square, rectangular, or circular in shape with surrounding walls that prevent escape. The most basic and common outcome of interest is "movement", which can be influenced by motor output, exploratory drive, freezing or other fear-related behaviour, sickness, relative time in circadian cycle, among many other variables. Within this task distance moved, time spent moving, rearing, and change in activity over time are measured. These parameters will be assessed by analyzing recordings using an

automated tracking system that is able to report the mentioned measurements. Particular outcomes as defecation, centre time, and activity within the first 5 minutes are showing anxiety like behaviour.

2. Blood and brains: In this set up a subset of the animals after each of the 10-day blocks will be euthanized at a certain time point after the behavioural measurements. The brains of the animals will be dissected and blood will be collected. The collected brains will be used for analysis at the transcriptional level. We will analyze the mRNA levels of different transcripts by RNA sequencing and/or qPCR of candidate genes in brain regions associated with the behavioural phenotype (e.g. ACC/vmPFC/OFC/striatum). Candidate genes will be selected on the basis of genetics landscapes of ASD and ADHD as published by our group (Poelmans et al., 2011; Poelmans et al., 2013). The blood will be used for analyzing hormone and lipid levels as well as immunological parameters. Steroids are powerful signalling molecules regulating a variety of physiological processes such as cell proliferation, cell differentiation, and reproduction. Variations in steroid concentrations may hint at hormonal imbalances that might play a role in diseases such as ASD and ADHD. Therefore, the blood hormone levels will be analyzed by using ELISA assays. Also, OM-85 might change specific gut microbiota that e.g. improve glucose homeostasis, the sensitivity to leptin, and target enteroendocrine cell activity. Measuring the lipid levels of later to be determined specific lipids will therefore also be analyzed from the blood by using ELISA. Immunological assessment in the blood will be the final measurement. Candidate immunological markers chosen are specific for immune changes seen in ASD and ADHD and/or are found by Vifor Pharma in previous published and unpublished research (Pasquali *et al*. 2014).

3. Microbiome: The microbiome compositions from baseline and after a certain amount of doses of the treatment (10, 20 or 30) are analyzed from the collected droppings, as there is a need for surrogate outcome measures that are accessible (e.g. ratio of bacterial composition in the gut predicting behavioural traits) plus as a basis for target discovery (to ascertain if alterations in the gut microbiome could be viable therapeutic approach to ASD and ADHD). This will be done by using a validated method from [redacted]. From the droppings, we will examine primarily bacteria that alter monoamine signaling [redacted] influence impulsivity-compulsivity and attention. However, we will also seek to conduct broader sequencing (Illumina-based 16S ribosomal RNA sequencing assays) which will be conducted at [redacted]. By using the 16S ribosomal RNA sequencing there will be a profile of phylogenetic marker genes to show the bacterial variability of the microbiome in the different strains and between the different treatment groups. This will seek to discover if there are other bacterial clusters that are related to these phenotypes [redacted], which can be used for future therapeutic approaches.

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

In order to minimize the number of animals, the required numbers of animals in the pilot will be based on descriptions of variation of previous studies in mice, a power analysis and knowledge of our team. The use of the same animals for different protocols (behavioural, blood and mRNA

analysis) at the same site in a randomized design and assessment of all readouts proposed in the application will be carefully planned to be able to maximize the scientific return from each animals, using as least animals as needed. All conditions will be kept constant throughout training to reduce within group variability. Also, the behavioural and neurochemical testing will be based on a certain amount of animals per group, measured with a power analysis, to ensure quality data that will be informative for analysis between the treatments. The power analysis is based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures, e.g. treatment duration. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

Our strategy, as mentioned in the project proposal, is to understand microbiome differences in ASD and ADHD. Therefore, we have selected different mouse models for these disorders which all have a different origin in terms of disease aetiology and that they exhibit different symptomatology, furthermore, the mice offer a efficient and accessible model systems to address questions of the basics of the role of microbiome in the ASD and ADHD. Another reason for choosing the mouse models for this project is that researchers have argued that the mouse and humans have a fairly similar physiological and anatomical structure of the gut. The intestinal tract in both species is made up of anatomical organs which are the same. Furthermore, also the average ratio of gut and body surface area equal between the mouse and human (Nguyen, 2015). Until now, murine models have been used most often in the studies of the microbiome. Therefore, the knowledge of the mouse gastroenterology, genetics and immunology surpasses any other model. This makes the mouse the best choice with results that are the best translationable to humans. Besides that we do realize that there are limitations to the use of the selected mouse models, therefore, the ultimate conclusions will be made with caution.
The mouse models of ASD and ADHD planned to use in this research are the maternal valproate (VPA) mouse model for ASD which involved prenatal disturbance of neuronal development, the Dopamine Transporter knock-out (DAT KO) mouse model for ADHD which focuses on the dopamine system, and the BALB/cJ mouse model which has a genetic component (more details about the strains below).
For the pilot all strain animals will be bought from the same supplier as their control animals (Charles River or Jackson) to prevent differences between the companies in mouse model, environment and social experiences before arriving in the lab. At the start of the experiment the animals will be [redacted] old to make sure the animals are the same age as the animals that will be used during the higher cognitive measurements. For those tasks the animals need to be able to work on the operant touchscreen boxes. Animals younger than [redacted] are, according to experience of the lab, not able to perform the tasks yet.
Since many of the deficits described for ASD and ADHD in humans are specific to males, in which there are higher incidence rates of neurodevelopmental disorders relative to females, we decided to only use male animals in the experiments. This will lead to a better outcome as the symptoms are more present and the mechanisms behind the disorders might be sexe specific. Furthermore, by using only males we minimize the

variation within the groups and therefore the experiment can be carried out in smaller groups of animals. Using only males will also avoid gender related variations during the study that are observed before in our own and the Cambridge lab, such as avoiding the potential oestrus cycle-related performance variability in female rodents and increased male aggression when male rodents must be tested in the same apparatus as female rodents.
Based on animal experiments that were performed by our research group and literature, we expect to use a maximum of mice in the whole pilot study of 1020. This calculation is based on the following a priori power analysis that is conducted by the statistical program G*Power. When using an F-test (ANOVA) with a power (beta) of 0.80, significant level (alpha) of 0.05 and the effect size (f) of 0.5, the minimal number of animals for the pilot according to the power analysis is n=48. As we are comparing four different treatment groups during the pilot (no gavage, vehicle oral gavage, medium dose oral gavage (40 mg p.o.) and high dose oral gavage (80 mg p.o.)) it means that we have a n=12 per treatment group minimal to fully characterize and optimize measurements for the follow-up study. A couple of animals will be added to each group as fallouts are to be expected (maximal 10%) that would reduce the group number to n=10 in the worst scenario, thereby hampering the statistical power and the detection of relevant effects. These fallouts can be due to natural and/or induced causes (e.g. age, natural health issues or stress occurring during the longitudinal treatment period). By using the formula: N=n/(1-uitvalspercentage) this leads to a group size of max. 14 animals. When including all three duration groups (10, 20 and 30 days of treatment), this would mean a total of 168 mice are needed for the pilot study per mouse model. Besides that all three mouse models have their own control group from the same breeder as the model and which undergoes the same procedures as the model. This is to make sure there are no confounds in epigenetics. These can occur from background and events before arriving at the research institute in Nijmegen. This means that we are using 3 mouse models and idem amount of control groups, making the total of the pilot 6 x 168 = 1008 mice. Adding to this the planned oral gavage training on a maximum of 12 animals, the grand total for the pilot will be 1020 mice spread throughout the three chosen mouse models:
1. *Valproate (VPA) mouse model:*
Maternal valproate (VPA) exposure has permanent adverse effects upon neurological and behavioural development. C57/Bl6 mouse pups exposed in utero to VPA demonstrate difficulties in social interaction, altered ultrasonic vocalization communication, reversal learning as well as the differential expression of plasticity-related genes (also altered in human ASD)(Rinaldi *et al*. 2007; Roullet *et al*. 2010). In the current study, C57/Bl6 mouse pups at [redacted] will be studied (following prenatal exposure to VPA in utero).
2. *DAT-/- mouse model:*
The dopamine transporter knock-out mouse model is the specific model for ADHD, which do not effectively clear extracellular DA and exhibit a generally overactive dopaminergic state. The model demonstrates strong face, construct and predictive validity for ADHD, and spontaneous hyperactivity and impaired learning and memory. Recent studies have also demonstrated an impaired cliff-avoidance reaction in DAT-KO mice, indicating increased impulsivity. Unlike wild-type or heterozygous mice, DAT-/- mice are significantly more active in novel and home-cage environments and demonstrates learning and memory deficits in the 8-arm maze, novel object recognition and social food preference transmission tests (Yamashita *et al*. 2013; Gainetdinov *et al.* 1999; Wong *et al.* 2012). In the current study we will employ [redacted] old DAT-/- (on a C57/Bl6 background strain).

3. *BALB/cJ mouse model:* The Balb/cJ mouse model will be compared to the Balb/cByJ; these two substrains differ only with respect to a few copy number variations, but have a markedly different behavioural phenotype. The Balb/cJ (when compared to Balb/cByJ and/or C57Bl/6J) was found to not only shows relatively low levels of social interaction in various settings and across various stages of development, but also other phenotypes with possible relevance to autism and ADHD, including relatively high levels of anxiety and aggressive behaviours, large brain size, underdevelopment of the corpus callosum, impulsive choices, hyperactivity and low levels of brain serotonin (unpublished [redacted]; Brodkin *et al*. 2007; Jacome *et al.* 2011; Fairless *et al*. 2008 & 2012; Bielas I. 2014; Mazei-Robinson and Blakely 2006; Leo 2013; Otobe and Makine 2004).

Species	Origin	Maximum number of animals	Life stage
Mice BALB/cJ	Charles River	168	At start of experiment[redacted]
Mice BALB/cByJ	Charles River	168	At start of the experiment [redacted]
Mice C57Bl/6 (VPA+controls)	Charles River	336	At start of the experiment [redacted]
Mice DAT-/-	Jackson	168	At start of the experiment [redacted]
Mice C57Bl/6	Jackson	168	At start of the experiment [redacted]
Mice BALB/cJ	Charles River	12	[redacted] old when oral gavage training starts

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

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

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

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

Replacement:

The objective of this project is to gain a greater understanding of the complex pathophysiological processes that are responsible for the manifestation of clinical psychiatric disorders (e.g. ASD and ADHD) in humans. These processes will involve the interaction of multiple factors from genetics even as environmental and social experiences. These complexities can only be modelled realistically in an intact organism. Studies of psychiatric disorders in human patients are limited due to heterogeneous genetic background, medication effects, and limited post-mortem brain tissue of poor quality, especially for childhood psychiatric disorders like ASD and ADHD. The mouse model offers an efficient and accessible model system to address questions of fundamental mammalian significance. This particular research proposal involves the use of BALB/c mice because they provide an excellent model system for studying the basics of the role of microbiome in the disorders. Rodents are the lowest mammalian species that are suitable, and readily available, for these investigations. These kinds of tests cannot be performed in vitro, e.g. cell cultures or tissue.

Reduction:

Before starting the pilot a literature search will be performed to find all known information relevant for the experimental design. Additionally, the mentioned a priori power analysis (see statistical methods and choice of animal models) will be performed based on data from literature and previous experiments in our lab. This is to determine the minimum amount of animals needed. A reduction in the number of animals per group, caused by drop out of animals would reduce statistical power needed to demonstrate the effects described before, making it necessary to take this into account. Animal use will be limited to the minimum necessary for obtaining scientifically meaningful results with large specificity and power. The use of biological samples from single animals for combined behavioural, blood and mRNA analysis will be carefully planned so as to maximize the scientific return from each animal. All conditions will, furthermore, be kept constant throughout training to reduce within group variability. All behavioural and neurochemical testing will be based on the calculated animals per group to ensure quality data that will be informative for analysis between the four treatment groups and three durations of treatment.

Refinement:

The experiments will be carried out in mice because there are no alternatives to study in vivo behaviour in lower animal specious. Most experimental procedures will only cause moderate discomfort or less as the use of adequate oral gavage by trained personal and the behavioural procedures selected, minimizes harm or inconvenience to the animals. Also, by using methods that are well established and the experience of all researchers involved, the discomfort is as low as possible and the translational value of this type of studies has been shown previously by our own and the Cambridge lab. The use of anaesthesia and analgesics is in general not required and may, if necessary to apply, cause even more discomfort than the oral gavage or procedures itself. To avoid the requirement of the anaesthesia or analgesics, close monitoring of animals during behavioural

testing and in the home cage will be done to ensure no discomfort. Therefore, the general health of the animals will be monitored daily by the researcher and/or biotechnician. If necessary approved euthanasia of animals experiencing morbid injury or pain will be performed.

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

In order to prevent unnecessary discomfort or stress the general health of the animals will be checked daily by the researcher or the biotechnicians. In addition, handling the animals every day will minimize stress of the oral gavage and when the behavioural task is performed. When an animal shows signs of suffering, pain or fear, the responsible researcher will be contacted to decide on anaesthesia, analgesics and humane endpoints to reduce discomfort. In case of anxiety, the animals will be observed and handled with care. By taking only blood after euthanization, we make sure to reduce discomfort or side effects. Besides this, adverse effects are not expected from the oral gavage nor the simple battery tasks on the animal's health based on knowledge we have from previous research at our lab and by [redacted]. There are no expected environmental adverse effects.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

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

During the pilot study it is necessary to house the BALB/cJ and their controls (BALB/cByJ) individually. For fhe BALB/cJ animals, as they are known to be aggressive when housed together, it is advisable to be housed individually. This will minimize the stress of fighting. The other strains and their controls will be standard group housed as the aim of the experiment is to find differences in behaviour between treatment groups and not within groups on individual level.

For all animals water is available ad libitum, but after the baseline the weight of the animals will slowly be reduced to a percentage of 85-95% of the measured free-feeding weight by controlling the daily amount of food they are given. This means that the animals are on mild food restriction. The reason for this is that the pilot study is used to determine the optimal dose and treatment duration used for the second experiment that uses the operant touchscreen system to measure higher cognitive performances. In this behavioural touchscreen battery of tasks it is important to ensure that the animals are motivated to fulfil the tasks, making mild food restriction needed. The pilot should therefore be as comparable as possible to the conditions of the second experiment.

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?

[X] 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?

[X] No > Continue with question I.
[] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

[X] No > Justify why pain relieving methods will not be used.

H. Pain and pain relief

Oral gavage is not expected to be painful and will be performed as quickly as possible by an experienced employee. The behavioural tasks that will be performed are also not causing pain to the animals.

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

I. Other aspects compromising the welfare of the animals

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

In the pilot study occuring types of discomfort can be: stress and euthanasia.

Explain why these effects may emerge.

In the pilot study a mild food restriction is necessary to make the results as comparable as possible to the second experiment, in which higher cognition is measured. This can cause stress. Stress can also be caused by the handling of the animals, oral gavage, housing conditions and the behavioural measurements. [redacted]

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

To minimize the stress, the [redacted] of the animals will be switched. This means that the testing will be during their [redacted], when they are naturally active. Also, before the experiment starts the animals will be housed for a week in order to acclimatise to their new environment. The food restriction will be set by slowly reducing the amount of food. After food restriction is complete, the animals will be on a mild restriction only. This means that the animals are a little under the free feeding weights to motivate them. The planned experiment, including euthanization, will not cause any pain or adverse effects as it will be performed by an experienced researcher who has been trained to perform the study.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

To achieve interpretable behavioural results, it is important that all animals are healthy and do not show signs of stress or reduced well being during experimental testing. Animals will be checked daily for signs of sickness. Animals that show overt signs of sickness, infection or loss of body weight (more than 15%) or other signs of reduced well-being will be taken out of the experiment and euthanized with pentobarbital. We do not expect effects of the behavioural tasks on the wellbeing of the mice. The treatment with the bacterial extract is even so not expected to cause any problems. However, all animals will be monitored very closely every day to be sure to notice any negative problems that might arise. If any signs mentioned before are signalled the animal will be taken out of the experiment and will be euthanized.

Indicate the likely incidence.

We expect that minimal 98% of the animals will experience mild discomfort, because we will not perform harmful procedures or expect an adverse effect of the bacterial extract. This means that <2% of the animals might have unexpected moderate or severe discomfort due to for example stress or sickness.

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).

According to the EU-guidelines 98% of the animals the cumulative discomfort will be moderate because of the the combination of all the procedures planned. This includes the discomfort of individual housing for the BALB/cJ and their control group and the longitudinal oral gavage and testing. For a maximum of 2% of the animals the level of discomfort can rise up to severe discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

L. Method of killing

[] No

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

At the end of the experiment we will determine *ex vivo* changes for which we need the brains of the mice to assess the mechanisms by which OM-85 lead to possible structural and functional changes in specific brain regions. Even so, the blood and possibly a piece of the intestine will be further researched after euthanizing the animal.

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

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

[X] Yes

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'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 2	Type of animal procedure Behaviour in ASD and ADHD models with [redacted]

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.

Behaviour in ASD and ADHD models with OM-85: Simple and higher cognitive measurements

The aim of the experiment is to evaluate the effect of the microbiome on higher cognitive behaviour by examining the effect of dietary intake of an immunological bacterial extract that modulates immune/inflammation gut responses in mouse models of ASD and ADHD. For this purpose, we plan to study the microbiome in animal models for ASD and ADHD and their controls, by means of faecal sample collection of the mice, under baseline conditions and subsequently treating the animals during a certain time with an immunological bacterial extract, called OM-85. The length of this and the dose will be decided on from the pilot study and can be different for each strain. In addition, during the treatment, simple and higher cognitive behaviour will be assessed by the use of simple battery tasks and operant chamber touchscreen tasks. At the end of the treatment animals will be scanned and sacrificed to assess the intestine/caecum and brain for changes.

The approach to assess the effects of the immunomodulatory bacterial extract [redacted] in mouse models of ASD and ADHD on brain, behaviour and cognitive function includes the following experiments in the same cohort of mice:

1. Simple battery tests: determine which effect the chosen dose of the [redacted] extract has on the simpler behaviour, the microbiome, intestine and the brain.
2. Higher cognitive tests: determine which effect the chosen dose of the [redacted] extract has on the more complex behaviour, the microbiome, intestine and the brain.

To determine the effects in of the treatment in both easy and complex behaviour we will use the following primary end points that have successfully been used in previous studies performed by our research group and by other international research groups [redacted]

- Treatment will be performed on the timepoint that differences in behaviour are most expected, which is known from previous experience [redacted]
- The effects of [redacted] treatment on the microbiome and therefore on the immune system and the brain and behaviour will be determined by assessing changes in multiple parameters:

1. Assessing simple behaviour:

In this set up the animals will perform three simple battery tasks to assess symptomatic behaviour of ASD and ADHD in the different strains during treatment with [redacted] All three tasks are easy to perform and it does not require long training time. The first task performed will be the Y-maze

Spontaneous Alternation task. This is a behavioural test for measuring compulsivity via the willingness of rodents to explore new environments. Over the course of multiple arm entries, the subject should show a tendency to enter a less recently visited arm. The number of arm entries and the number of triads are recorded in order to calculate the percentage of alternation. The second test will be the Open Field. The Open Field task is a simple sensorimotor test used to determine outcomes as: general activity levels, gross locomotor activity, and exploration habits in rodent models of CNS disorders. These parameters will be assessed by analyzing recordings using an automated tracking system that is able to report: time spend in centre and pre-defined zones, overall mobility, distance moved and velocity within the first 5 minutes. The final third tasks will be the elevated plus maze. The elevated plus maze in a behavioural task performed in animal models as a general tool in neurobiological anxiety research. The animal's aversion to open spaces and tendency to be close to vertical surfaces is videotaped and expressed as the time the animals is spending more in the enclosed arms of the maze.

2. Assessing higher cognitive behaviour: For higher cognitive measures in the mouse models we will administer tests in the operant touchscreen chamber during treatment with [redacted] These are analogous to the Cambridge Neuropsychological Test Automated Battery (CANTAB), which is a computer tablet/touchscreen-based cognitive battery that can assess changes in behaviour across different cognitive domains. For measuring impulsivity, compulsivity and inattention in the animal models a couple of tasks will be performed. [redacted] For the animals tested on ADHD symptoms, a translational tasks of attention and impulsivity [redacted]

[redacted]

[redacted]

[redacted]

[redacted]

3. Scans and transcriptional analysis of the brain: In this set up the animals will be performing the behavioural tasks after which they will be scanned and euthanized. We will apply similar MRI protocols as in humans during scanning. This includes DTI as well as resting state functional MRI (rs-fMRI). These techniques have been successfully employed in the 11.7T MRI scanner [redacted] We will also focus on frontostriatal circuits, [redacted] Importantly, our MRI approach will enable standardized and well-controlled assessment of neural underpinnings [redacted] This will provide relevant translational information for the human/clinical MRI studies that will be performed later by the same researchers. After scanning, the brains of the animals will be dissected after collection. The collected brains will be used for analysis at the transcriptional level. We will analyze the mRNA levels of different transcripts by RNA sequencing and/or qPCR of candidate genes in brain regions associated with the behavioural phenotype (e.g. ACC/vmPFC/OFC/striatum). Candidate genes will be selected on the basis of genetics landscapes of ASD and ADHD as published by our group (Poelmans et al., 2011; Poelmans et al., 2013) and from the results from the pilot study.

4. Microbiome and caecum:

The microbiome compositions from the distal gut microbiome is typically assayed via stool, because of the non-invasive route of collection, and have provided important insights into the distal gut microbiome composition and function. Therefore, the microbiome baseline and after a certain amount of doses of the treatment are analyzed from collected droppings. The collection will be done by using a validated method from [redacted] From the droppings, we will examine primarily bacteria that alter monoamine signaling as the [redacted] However, we will also seek to conduct broader sequencing (Illumina-based 16S ribosomal RNA sequencing assays) which will be conducted at [redacted] By using the 16S ribosomal RNA sequencing there will be a profile of phylogenetic marker genes to show the bacterial variability of the microbiome at different time points, in the different strains and between the different treatment groups. This will seek to discover if there are other bacterial clusters that are related to these phenotypes beyond [redacted], which can be used for future therapeutic approaches. Besides the analysis of the fecal material, it is important to recognize that adherent microbiota residing on mucosal surfaces of the gut may be vastly different from those present in fecal material, as different compartments of the gut may be locally colonized by distinct communities of bacteria (Mulle, 2013). Therefore, the caecum is harvested and weighed and will be used to detect lumen-associated and mucus-associated bacterial species. The analysis will be performed by the same external company as the faecal material.

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

Behaviour in ASD and ADHD models with OM-85: Simple and higher cognitive measurements

A second experiment will be performed on the same ASD and ADHD animal models (BALB/cJ, VPA and DAT KO) and their controls. During this second experiment the animals will receive the [redacted] extract [redacted] to be able to measure behavioural changes in simple and higher cognitive measurements. From the pilot study we will know per strain which dose and duration has to be given for the highest changes. This information will be used to decide on the intervention time point and duration for each strain while performing simple and higher cognitive behavioural tasks.

During the second experiment we will start by collecting microbiome at [redacted] This will be done by collecting faeces of each animal using a validated work protocol received from [redacted] After that the animals are starting with the simple battery tasks, followed by the touchscreen tasks. However, before starting the behavioural measurements, the weight of the animals will be slowly reduced to a percentage of 85-95% of the measured free-feeding weight by controlling the daily amount of food they are given (~2–5 g food per 25–35 g of body weight). This way the animals are subjected to a mild food restriction before the onset of the tasks, and continuing throughout the course of the experiment. Food restriction is implemented for motivational reasons.

1. Behavioural measurements:
simple and higher cognitive measurements

Simple cognitive testing: The first simple battery task will be the Y-maze: This is a behavioural test for measuring compulsivity via the willingness of rodents to explore new environments as rodents tend to alternate arm visits and naturally avoid repeated entries ('spontaneous alternation'). This is reflecting natural exploration behaviour of a novel environment. In order to determine spontaneous alternation, mice will be placed in the long arm of a Y-maze and are allowed to explore the maze for 5 minutes. Over the course of multiple arm entries, the subject should show a tendency to enter a less recently visited arm. The number of unique arm entries and the number of triads are recorded in order to determine the percentage of spontaneous alternation behaviour.

Spontaneous alternation in the Y-maze will be assessed according to e.g. the Nature Protocols paper on spontaneous alternation in the T-maze by Deacon and Rawlins (2006).

The second simple battery task will be the Open Field test. The open field test (OFT) is a common simple measure of sensorimotor performance. It is used to determine outcomes as: general activity levels, gross locomotor activity, and exploration habits in rodent models of CNS disorders (Gould *et al.* 2009). Both the quality and quantity of the activity can be measured. Principally, the open field (OF) is an enclosure, generally square, rectangular, or circular in shape with surrounding walls that prevent escape. The most basic and common outcome of interest is "movement", which can be influenced by motor output, exploratory drive, freezing or other fear-related behaviour, sickness, relative time in circadian cycle, among many other variables. Within this task distance moved, time spent moving, rearing, and change in activity over time are measured. These parameters will

be assessed by analyzing recordings using an automated tracking system that is able to report the mentioned measurements. Particular outcomes as defecation, centre time, and activity within the first 5 minutes are showing anxiety like behaviour.

The final, third simple battery task will be the elevated plus maze. The elevated plus maze (EPM) is a widely used behavioural assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents and steroid hormones, and to define brain regions and mechanisms underlying anxiety-related behaviour (Walf and Frye 2007). The task is based on rodents' aversion of open spaces. This aversion leads to behaviour in which avoidance of open areas is involved by confining movements to enclosed spaces or the edges of a bounded space. The EPM test consists of a plus-shaped apparatus with two open and two enclosed arms. Each arm has an open roof and is elevated from the floor. Briefly, the mice are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer simultaneously for 5 min. A reduction in anxiety is indicated in the EPM by an increase in the proportion of time spent in the open arms, and an increase in the proportion of entries into the open arms. The total number of arm entries and number of closed-arm entries are sometimes also used as a measurement of general activity (Lalonde and Strazielle 2008). Other ethological parameters (i.e., rears, head dips and stretched-attend postures) can also be observed. An increase in open arm activity (duration and/or entries) reflects anti-anxiety behaviour during the one session each animal will get.

After these behavioural measurements the animals will be trained for the operant touchscreen chambers for higher cognitive behavioural tasks.

Higher cognitive testing:

Cognitive measurements using touchscreen operant chambers

The following two touchscreen experiments will be executed according to the most recent protocol provided by Campden Instruments Ltd and Nature Protocols papers (Mar *et al.* 2013*;* Horner *et al*. 2013; Oomen *et al*. 2013) and are executed in the Bussey Mouse Touch Screen Chambers, Campden Instruments Ltd. Both the main researcher and the substitute researcher have gained experience using this method in the lab of Tim Bussey and Lisa Saksida (Cambridge) and will have access to the most recent updates and developments regarding this method.

1. Visual Discrimination Reversal Learning Visual discrimination reversal learning (VDRL) is a measure of cognitive flexibility and an important cognitive "execution function". Successful reversal allows an individual to flexibly adjust their behaviour to changing environments and rules. In order to assess this, appetitive reversal learning protocols are widely used. In such paradigms, subjects are taught to discriminate and choose between a rewarded and unrewarded stimulus and after the acquisition of such an association, stimulus-reward contingencies are reversed. The rate and extend to which the reversed association is acquired provides an index of flexibility. This approach has been successfully applied in the touchscreen operant platform (Bussey *et al.* 2008 ; Mar *et al.* 2013) and is currently successfully used in the Glennon lab as well. Of interest, ASD is characterized by a reduced cognitive flexibility, which may result in reduced learning in the reversal learning paradigm. In the existing reversal protocol, animals are mildly punished by a time-out [redacted] and the presence of a light in the chamber. Reversal learning is assessed by pretraining animals in touchscreen platform. The pretraining of the mice will start with a series of habituation sessions [redacted] in which the mice learn to collect the food reward [redacted] with the addition of "punish incorrect" phase. In this phase, which is performed before the start of most touchscreen tests, animals are punished for touching the blank location by a time-out and light-on, to prevent the development of a

side-bias in the chamber. These sessions continue until the mice have collected all rewards (approximately 2-4 weeks). Then, the mice will proceed to the initial touchscreen training. In all phases animals are subjected to mild food restriction beginning before the pretask training and continuing throughout the course of the experiment to ensure sufficient levels of motivation during the operant box task. To achieve the food restriction, the weight of the animals will be reduced slowly to a percentage of 85-95% of the measured free-feeding weight by controlling the daily amount of food they are given (~2–5 g food per 25–35 g of body weight). In order to assess Reversal, animals are first trained to associate a stimulus with a reward. During daily sessions [redacted] two stimuli are presented (S+ and S-) on each trial. These are randomly assigned to the two locations on the screen. After animals reach criterion [redacted] on two consecutive days, reward contingencies are reversed and re-acquisition of the new associations is started. Based on earlier work, animals are estimated to acquire the task in 8-10 days. Reversal is usually acquired in 13-16 sessions (reversing contingencies with the same stimuli).

At the optimum intervention time point, defined by the results from the pilot, the animals receive a vehicle (group 1) or a dose of the bacterial dietary [redacted] (group 2), possibly while they are (partly) performing the reversal learning behavioural task**.** Thereafter the animals will be re-tested on the stimuli to make sure the mice still reach criterion. When reaching criterion 2 days in a row the reversal starts and the animals will have to switch responding to the new S+ (previously unrewarded) and S- (previously rewarded).

2. Extinction task

The second touchscreen task that will be performed in the same cohort of animals is the extinction task. This task is testing the ability to learn to stop making a response that no longer provides a desired or adaptive outcome (compulsivity). It can be as important as learning to produce behaviour in the first place. Since early descriptions of extinction, it has become clear that it is a complex phenomenon: in many instances, behavioural changes cannot be simply explained by forgetting what has previously been learned, and its expression has been demonstrated to be highly sensitive to context (Horner *et al.* 2013*)*. In the extinction procedure outlined by the Bussey and Saksida lab (Cambridge), animals are first trained to acquire a simple visually guided response (e.g. touch a white square) in order to earn a reward. After this acquisition phase, animals are presented with similar opportunities to respond, but in the absence of rewards and associated cues. The time it takes and the extent to which animals suppress their responding provide the basic indices of extinction learning. Variants of this protocol have been used to test extinction in various mutant mouse lines and genetic strains. Touchscreen extinction assays have been effectively used to phenotypically characterize behavioural differences between inbred mouse strains. These studies have shown that some strains of mice, including the BALB/cJ mice, perform well on the extinction task compared with certain other strains (unpublished data Glennon lab; Hefner *et al*. 2008; Norcross *et al*. 2008).

During the response acquisition phase, sessions begin with a free reward delivery to the magazine and magazine light illumination, indicating that a trial may be initiated. Trials are initiated by the animal's head entry into the magazine (turning off the magazine light and activating a 0.2-s auditory click), in which subsequent head withdrawal from the magazine initiates presentation of a single, solid white square stimulus at a central location on the touchscreen. When the animal touches the stimulus, the stimulus is removed from the screen, a reward is delivered and the magazine light and a 1-s tone are turned on. Following reward collection, the magazine light is extinguished and a 5-s ITI commences, after which the magazine light is illuminated and animals are again given the opportunity to initiate a new trial.

In the extinction phase of the current procedure, each trial typically begins with a 10-s ITI, after which the single, solid white square stimulus is presented on the touchscreen. The animal is not required to initiate the trial. If the animal either touches the stimulus (response) or does not touch the stimulus within a 10-s duration (omission), the stimulus is removed and the 10-s ITI leading to the next trial is initiated. No rewards or conditioned reinforcers (e.g., tray light or tone associated with reward delivery) are delivered during the extinction phase.

Animals typically require multiple sessions to achieve criterion in extinction. Various post-training probe tests may be carried out after the extinction phase [redacted] to further assess aspects of extinction learning that may be amenable to acute experimental manipulations. The most well-established post-extinction probes include various forms of relapse—the reoccurrence of the relevant behavior learned before extinction training. For example, reacquisition may be assessed by examining recovery of pre-extinction behavior after the unconditioned reinforcer (i.e., food reward) and/or conditioned reinforcers (i.e., magazine light and brief tone) are reintroduced in a manner fully contingent on presentation of, and the animal's response to, the visual stimulus (i.e., identical to the learning stage before removal of these reinforcers during extinction). These post-extinction probes may be used to evaluate propensity to relapse and also the nature and extent of extinction processes; for example, the strength of reinstatement is believed to be an indicator of the strength of contextual conditioning.

If necessary, again depending on the results from the pilot, the animals receive the different treatments before/during the extinction tasks. Including a vehicle (group 1), or a dose of the bacterial extract [redacted] (group 2)**.**

3. 5-choice serial reaction time task (5-CSRTT**)** OR continuous performance task (CPT)

The 5-choice serial reaction time task is a measure of attention and impulse control. This task will be conducted according to previous publications (Humby *et al.* 2005, Mar *et al.* 2013*)*. The pretraining of the mice will start with a series of habituation sessions (called shaping) in which the mice learn to collect the food reward (condensed strawberry flavoured milk). These sessions continue until the mice have collected all rewards. Then, the mice will proceed to the initial touchscreen training. During this phase, the association between stimuli presented on the touchscreen and the delivery of the reward is introduced. For this, stimuli are presented on the screen for 30 sec.; removal of the stimulus coincided with delivery of reward. If the mouse touches the stimulus during this period, it will receive (a larger) reward instantly. The criterion is 30 trials in 30 minutes. The next stage is called 'must touch' during which the animal is required to touch the screen in order to obtain a reward. Finally, animals are learned to 'initiate' trials; triggering stimulus presentation by a nose poke into the food magazine. After pretraining, animals are trained on the task proper. During this, mice are taught to touch the stimulus presented in one of the 5 locations either during presentation or during a short period following stimulus presentation, the so-called limited-hold period, in order to obtain a reward. There is no response if the mouse touches one of the other four stimulus locations. 5-CSRT training will continue until animals reach baseline performance, in which the mouse needs to respond to the stimulus within a limited time period and perform with >80% accuracy and <20% omissions on two consecutive sessions (one session per day, max. 1 hour). There are typically 100 trials in a session, which might last for up to 60 min. Again, this training stage might last between 15 and 30 days untill the mice reach the basline levels of more than 80% accuracy and less than 20% omissions for two consecutive days.

In the probe sessions, attentional functioning and measures of impulsivity can be assessed by manipulating the basic task parameters. Usually, the type of manipulations are as follows:

1. Altering the duration of the inter-trial interval, which increases the attentional load by disrupting the temporal predictability of the stimulus onset.
2. Reducing the stimulus duration
3. Reducing the stimulus brightness
4. Imposition of a burst of distracting white noise following the beginning of the inter-trial interval
5. Combination of the above.

The design and timing of each of these probe sessions will be dependent on the performance of the animals, and decisions may have to be made ad-hoc every day. For example, once the performance of the animals has stabilized at criteria with the baseline parameters, attentional functioning can be assessed by manipulating the basic task parameters, but performance on such conditions cannot be fully predicted beforehand, therefore the number of sessions needed to test animals on these parameters may vary between 1 to 6 per condition. It may be necessary to optimize the

parameters of such manipulations (such as precise determination of stimulus duration, ITI etc), which may add to the number of sessions needed per probe. In addition, it is custom to 'rebaseline' animals on the standard test-conditions in between these so-called probe sessions, to prevent confounding transfer effects of probe-conditions into the next.
At the optimum intervention time point, defined by the results from the pilot, the animals receive a vehicle (group 1) or a dose of the bacterial dietary [redacted] (group 2), possibly while they are (partly) performing the 5-choice behavioural task**.** Also, as with the other experiments, the animals will be mildly food restricted to ensure motivation. Every manipulation can take 1-21 sessions [redacted] although expected to last an average of 4 sessions (personal experience from the lab in Cambridge). Therefore, the total duration of this stage of the experiment is highly variable, ranging from a few weeks to months (depending on the number of manipulations that need to be tested). This means that we can only give an estimate of the maximum time that animals need to complete the experiment, which is -in total maximal 7 months and includes pretraining, training to baseline and testing.

Like the 5-choice serial reaction task, the continuous performance task requires the rodent to respond to a brief visual stimulus. However, in this task, stimuli appear in sequence in only one location and the animal has to learn to respond only to the correct stimulus, but not to other (unrewarded) stimuli. Therefore, this "go/no-go" task measures both attentional and inhibitory systems within a single task paradigm, enabling the assessment of vigilance. [redacted]

2. Scans and transcriptional analysis of the brain: To identify neural correlates of impulsivity, compulsivity and inattention [redacted] we will apply state-of-the-art MRI acquisition and analysis methods enabling serial assessment of whole-mouse brain structural and functional neuronal networks in the BALB/cJ, VPA and DAT-/- mouse models of ASD and ADHD. For optimal translation to the human neuroimaging studies in this project, we will apply similar MRI protocols, including DTI as well as resting state functional MRI (rs-fMRI). These techniques have been successfully employed in the 11.7T MRI scanner [redacted] We will also focus on frontostriatal circuits, namely those originating from the anterior cingulate cortex (ACC) and orbitofrontal cortex (OFC). Importantly, our MRI approach will enable standardized and well-controlled assessment of neural underpinnings in response to [redacted] This will provide relevant translational information for the human/clinical MRI studies.

During the scanning, the animals will be anesthetized and we will acquire and analyze serial measurements per session in order to measure changes in global and regional brain tissue volumes, integrity of white matter connections, and functional connectivity between grey matter regions-of-interest in the fronto-striatal circuit of normal C57/Bl6 and VPA mice and DAT-/- mice (both on a C57/Bl6 background) and the BALB/cJ and their controls (BALB/cByJ)
The serial scan will contain:
a) 3D volumetric MRI: Volumetric imaging is a 3D technique that permits in vivo quantification of brain compartment volume, and has many applications in cognitive, clinical and comparative neuroscience. In this method all the signals of MRI are collected from the entire tissue samples. It will image the whole brain, therefore providing a high multiplanar reconstruction in all planes (Keller and Roberts 2010).
b) Diffusion Tensor Imaging (DTI) / Diffusion Kurtosis Imaging (DKI): Diffusion tensor imaging (DTI) is a sophisticated form of diffusion weighed imaging which allows for the determination of directionality as well as the magnitude of water diffusion in the brain in order to produce neural tract

images instead of using this data solely for the purpose of assigning contrast or colors to pixels in a cross sectional image (Manenti *et al*. 2007). This kind of MRI enables to visualize white matter fibers in the brain and can map subtle changes in the white matter associated with diseases where the brain's wiring is abnormal, such as ASD and ADHD. DKI provides independent and complementary information to that acquired with traditional diffusion techniques. The additional information is thought to indicate the complexity of the microstructural environment of the imaged brain tissue (Steven *et al.* 2014).

c) resting state functional MRI (rs-fMRI): is a method of functional brain imaging that can be used to evaluate regional interactions that occur when a subject is not performing an explicit task (Biswal 2012). This resting brain activity is observed through changes in blood flow in the brain which creates what is referred to as a blood-oxygen-level dependent (BOLD) signal that can be measured using functional functional Magnetic Resonance Imaging (fMRI) (Cole *et al*. 2010).

After scanning, the animals will be euthanized and the brains of the animals will be collected. The collected brains will be used for analysis at the transcriptional level. We will analyze the mRNA levels of different transcripts by RNA sequencing and/or qPCR of candidate genes in brain regions associated with the behavioural phenotype [redacted] Candidate genes will be selected on the basis of genetics landscapes of ASD and ADHD as published by our group [redacted] and from the results from the pilot study.

3. Microbiome and caecum: The microbiome compositions from the distal gut microbiome is typically assayed via stool, because of the non-invasive route of collection, and have provided important insights into the distal gut microbiome composition and function. Therefore, the microbiome baseline and after a certain amount of doses of the treatment are analyzed from collected droppings. The collection will be done by using a validated method from Prof. Cryan of University College of Cork. From the droppings, we will examine primarily bacteria that alter monoamine signaling [redacted] However, we will also seek to conduct broader sequencing (Illumina-based 16S ribosomal RNA sequencing assays) which will be conducted at [redacted] By using the 16S ribosomal RNA sequencing there will be a profile of phylogenetic marker genes to show the bacterial variability of the microbiome at different time points, in the different strains and between the different treatment groups. This will seek to discover if there are other bacterial clusters that are related to these phenotypes [redacted], which can be used for future therapeutic approaches. Besides the analysis of the fecal material, it is important to recognize that adherent microbiota residing on mucosal surfaces of the gut may be vastly different from those present in fecal material; furthermore, different compartments of the gut may be locally colonized by distinct communities of bacteria (Mulle, 2013). Therefore, the caecum is harvested and weighed and will be used to detect lumen-associated and mucus-associated bacterial species. The analysis will be performed by the same external company as the faecal material.

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

In order to minimize the number of animals, the required numbers of animals in the behavioural experiment will be based on descriptions of variation of previous studies in mice, a power analysis and knowledge of our team. The use of the same animals for different protocols (behavioural, scanning and brain analysis) at the same site in a randomized design and assessment of all readouts proposed in the application will be carefully planned to be able to maximize the scientific return from each animals, using as least animals as needed. All conditions will be kept constant throughout training to reduce within group variability. Also, the behavioural and neurochemical testing will be based on a certain amount of animals per group, measured with a power analysis, to ensure quality data that will be informative for analysis between the treatments. The power analysis is based on data from literature and previous experiments to determine the minimum amount of animals needed. We will also take into account drop out of animals during the experiment due to experimental procedures. If necessary, a biostatistician will be consulted to assist in these power calculations.

B. The animals

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

Our strategy, as mentioned in the project proposal, is to understand microbiome differences in ASD and ADHD. Therefore, we have selected different mouse models for these disorders which all have a different origin in terms of disease aetiology and that they exhibit different symptomatology (ASD, ADHD or comorbid), furthermore, the mice offer an efficient and accessible model systems to address questions of the basics of the role of microbiome in the ASD and ADHD. Another reason for choosing the mouse models for this project is that researchers have argued that the mouse and humans have a fairly similar physiological and anatomical structure of the gut. The intestinal tract in both species is made up of anatomical organs which are the same. Furthermore, also the average ratio of gut and body surface area equal between the mouse and human (Nguyen, 2015). Until now, murine models have been used most often in the studies of the microbiome. Therefore, the knowledge of the mouse gastroenterology, genetics and immunology surpasses any other model. This makes the mouse the best choice with results that are the best translationable to humans. Besides that we do realize that there are limitations to the use of the selected mouse models, therefore, the ultimate conclusions will be made with caution.
The mouse models of ASD and ADHD planned to use in this research are the maternal valproate (VPA) mouse model for ASD which involved prenatal disturbance of neuronal development, the Dopamine Transporter knock-out (DAT KO) mouse model for ADHD which focuses on the dopamine system, and the BALB/cJ mouse model which has a genetic component (more details about the strains below).
For the behavioural second experiment all strain animals will be bought from the same supplier as their control animals (Charles River or Jackson) to prevent differences between the companies in mouse model, environment, social experiences and microbiome before arriving in the lab. At the start of the experiment the animals will be 8 weeks old to make sure the animals are the same age as the animals that will be used during the higher cognitive measurements. For those tasks the animals need to be able to work on the operant touchscreen boxes. Animals younger than 8 weeks are, according to experience of the lab, not able to perform the tasks yet.

Since many of the deficits described for ASD and ADHD in humans are specific to males, in which there are higher incidence rates of neurodevelopmental disorders relative to females, we decided to only use male animals in the experiments. This will lead to a better outcome as the symptoms are more present and the mechanisms behind the disorders might be sexe specific. Furthermore, by using only males we minimize the variation within the groups and therefore the experiment can be carried out in smaller groups of animals. Using only males will also avoid gender related variations during the study that are observed before in our own and the Cambridge lab, such as avoiding the potential oestrus cycle-related performance variability in female rodents and increased male aggression when male rodents must be tested in the same apparatus as female rodents.

Based on animal experiments that were performed by our research group and literature, we expect to use a maximum of mice in the second experimental study of 120. This calculation is based on the following a priori power analysis that is conducted by the statistical program G*Power. When using an F-test [redacted] with a power [redacted] of 0.80, significant level [redacted] of 0.05 and the effect size [redacted] of 0.5, the minimal number of animals for the pilot according to the power analysis is n=34. As we are comparing two different treatment groups during the behavioural experiments per strain (vehicle oral gavage, dose of OM-85 oral gavage) it means that we have a n=17 per treatment group minimal to fully characterize and optimize measurements for the follow-up study. A couple of animals will be added to each group as fallouts are to be expected (maximal 15%) that would reduce the group number to n=15 in the worst scenario, thereby hampering the statistical power and the detection of relevant effects. These fallouts can be due to natural and/or induced causes (e.g. age, natural health issues or stress occurring during the behavioural testing period, not being able to learn the cognitive touchscreen tasks). By using the formula: N=n/(1-uitvalspercentage) this leads to a group size of max. 20 animals. As we are using 3 mouse models and idem amount of control groups, the total of the pilot will be 6 x 20 = 120.

Making this the grand total for the second experiment, spread throughout the three chosen mouse models:

1. Valproate (VPA) mouse model:

Maternal valproate (VPA) exposure has permanent adverse effects upon neurological and behavioural development. C57/Bl6 mouse pups exposed in utero to VPA demonstrate difficulties in social interaction, altered ultrasonic vocalization communication, reversal learning as well as the differential expression of plasticity-related genes (also altered in human ASD)(Rinaldi et al. 2007; Roullet et al. 2010). In the current study, C57/Bl6 mouse pups at [redacted] will be studied (following prenatal exposure to VPA in utero and a sham exposure in the controls).

2. DAT-/- mouse model:

The dopamine transporter knock-out mouse model is the specific model for ADHD, which do not effectively clear extracellular DA and exhibit a generally overactive dopaminergic state. The model demonstrates strong face, construct and predictive validity for ADHD, and spontaneous hyperactivity and impaired learning and memory. Recent studies have also demonstrated an impaired cliff-avoidance reaction in DAT-KO mice, indicating increased impulsivity. Unlike wild-type or heterozygous mice, DAT-/- mice are significantly more active in novel and home-cage environments and demonstrates learning and memory deficits in the 8-arm maze, novel object recognition and social food preference transmission tests (Yamashita et al. 2013; Gainetdinov et al. 1999; Wong et al. 2012). In the current study we will employ [redacted] old DAT-/- (on a C57/Bl6 background strain).

3. BALB/cJ mouse model: The Balb/cJ mouse model will be compared to the Balb/cByJ; these two substrains differ only with respect to a few copy number variations, but have a markedly different behavioural phenotype. The Balb/cJ (when compared to Balb/cByJ and/or C57Bl/6J) was found to not only shows relatively low levels of social interaction in various settings and across various stages of development, but also other phenotypes with

possible relevance to autism and ADHD, including relatively high levels of anxiety and aggressive behaviours, large brain size, underdevelopment of the corpus callosum, impulsive choices, hyperactivity and low levels of brain serotonin (unpublished [redacted] Brodkin et al. 2007; Jacome et al. 2011; Fairless et al. 2008 & 2012; Bielas I. 2014; Mazei-Robinson and Blakely 2006; Leo 2013; Otobe and Makine 2004).

Species	Origin	Maximum number of animals	Life stage
Mice BALB/cJ	Charles River	20	At start of experiment 8 weeks
Mice C57/Bl6 (VPA+controls)	Charles River	40	At start of experiment 8 weeks
Mice BALB/cByJ	Charles River	20	At start of experiment 8 weeks
Mice DAT KO	Jackson	20	At start of experiment 8 weeks
Mice C57/Bl6	Jackson	20	At start of experiment 8 weeks

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.
[] Yes > Explain why re-use is considered acceptable for this animal procedure.

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

[] No
[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

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

Replacement:

The objective of this project is to gain a greater understanding of the complex pathophysiological processes that are responsible for the manifestation of clinical psychiatric disorders (e.g. ASD and ADHD) in humans. These processes will involve the interaction of multiple factors from genetics even

as environmental and social experiences. These complexities can only be modelled realistically in an intact organism. Studies of psychiatric disorders in human patients are limited due to heterogeneous genetic background, medication effects, and limited post-mortem brain tissue of poor quality, especially for childhood psychiatric disorders like ASD and ADHD. The mouse models chosen offer efficient and accessible model systems to address questions of fundamental mammalian significance in the disorders of ASD and ADHD. This particular research proposal involves the use of VPA, DAT KO and BALB/c mice because they provide the basics of the role of microbiome in the disorders. Rodents are the lowest mammalian species that are suitable, and readily available, for these investigations. These kinds of tests cannot be performed in vitro, e.g. cell cultures or tissue.

Reduction:
Before starting the second behavioural tests a literature search will be performed to find all known information relevant for the experimental design. Additionally, the mentioned a priori power analysis (see statistical methods and choice of animal models) will be performed based on data from literature and previous experiments in our lab. Of course the data from the pilot study will, moreover, reveal the necessary information about the amount of animals necessary to use and some of the basics of the role of microbiome in the disorders. A reduction in the number of animals per group, caused by drop out of animals would reduce statistical power needed to demonstrate the effects described before, making it necessary to take this into account. Animal use will be limited to the minimum necessary for obtaining scientifically meaningful results with large specificity and power. The use of biological samples from single animals for combined behavioural, brain, caecum and scans analysis will be carefully planned so as to maximize the scientific return from each animal. All conditions will, furthermore, be kept constant throughout training to reduce within group variability. All behavioural and neurochemical testing will be based on the calculated animals per group to ensure quality data that will be informative for analysis between the four treatment groups and three durations of treatment.

Refinement:
The experiments will be carried out in mice because there are no alternatives to study in vivo behaviour in lower animal specious. Most experimental procedures will only cause moderate discomfort or less as the use of adequate oral gavage by trained personal and the behavioural procedures selected, minimizes harm or inconvenience to the animals. Also, by using methods that are well established and the experience of all researchers involved, the discomfort is as low as possible and the translational value of this type of studies has been shown previously by our own and the Bussey-Sakside lab in Cambridge. The use of analgesics is in general not required and may, if necessary to apply, cause even more discomfort than the oral gavage or behavioural procedures itself. To avoid the requirement of the analgesics, close monitoring of animals during behavioural testing and in the home cage will be done to ensure no discomfort. Therefore, the general health of the animals will be monitored daily by the researcher and/or biotechnician. If necessary approved euthanasia of animals experiencing morbid injury or pain will be performed. During scanning the animals will be anaesthetized to be able to get the serial MRI's. Before the animals wake up, they will be euthanized, preventing any side-effects from the anaesthesia and keeping discomfort as low as possible.

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

In order to prevent unnecessary discomfort or stress the general health of the animals will be checked daily by the researcher or the biotechnicians. In addition, handling the animals every day will minimize stress of the oral gavage and when the behavioural task is performed. When an animal shows signs of suffering, pain or fear, the responsible researcher will be contacted to decide on analgesics and humane endpoints to reduce discomfort. In case of anxiety, the animals will be observed and handled with care. For the brainscanning it is necessary to use anaesthesia. This might influence the brain and the behaviour in a negative way when waking up afterwards. To prevent this, and reduce the discomfort or possible side effects of the anaesthesia, the animals will be euthanized after scanning before they wake up, while their brains will be collected. Besides this, adverse effects are not expected from the oral gavage nor the simple battery tasks or higher cognitive touchscreen tasks on the animal's health based on knowledge we have from previous research at our lab and by Vifor Pharma. There are no expected environmental adverse effects.

Repetition and Duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

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

During the second, behavioural, study it is necessary to house the BALB/cJ and their controls (BALB/cByJ) individually. For fhe BALB/cJ animals, as they are known to be aggressive when housed together, it is advisable to be housed individually. This will minimize the stress of fighting. The other strains and their controls will be standard group housed as the aim of the experiment is to find differences in behaviour between treatment groups and not within groups on individual level.
For all animals water is available ad libitum, but after the baseline the weight of the animals will slowly be reduced to a percentage of 85-95% of the measured free-feeding weight by controlling the daily amount of food they are given. This means that the animals are on mild food restriction. The reason for this is that during the second experiment the operant touchscreen system is used to measure higher cognitive performances. In this behavioural touchscreen battery of tasks it is important to ensure that the animals are motivated to fulfil the tasks, making mild food restriction needed.

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?

[X] 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?

[X] No > Continue with question I.
[] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

Oral gavage is not expected to be painful and will be performed as quickly as possible by an experienced employee. The behavioural tasks that will be performed are also not causing pain to the animals.

Explain why these effects may emerge.

In the behavioural second study a mild food restriction is necessary to make the animals motivated during the performance of the tasks in which higher cognition is measured. This can cause stress, but is not expected to be causing adverse effects in any other way. Stress can also be caused by the handling of the animals, oral gavage, housing conditions and the behavioural measurements. [redacted]

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

To minimize the stress, the day and night cycle of the animals will be switched. This means that the testing will be during their night, when they are naturally active. Also, before the experiment starts the animals will be housed for a week in order to acclimatise to their new environment. The food restriction will be set by slowly reducing the amount of food. After food restriction is complete, the animals will be on a mild restriction only. This means that the animals are a little under the free feeding weights to motivate them. The planned experiment, including euthanization, will not cause any pain or adverse effects as it will be performed by an experienced researcher who has been trained to perform the study.

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.
[X] Yes > Describe the criteria that will be used to identify the humane endpoints.

To achieve interpretable behavioural results, it is important that all animals are healthy and do not show signs of stress or reduced well being during experimental testing. Animals will be checked daily for signs of sickness. Animals that show overt signs of sickness, infection or loss of body weight (more than 15%) or other signs of reduced well-being will be taken out of the experiment and euthanized with pentobarbital. We do not expect effects of the behavioural tasks on the wellbeing of the mice. The treatment with the bacterial extract is even so not expected to cause any problems. However, all animals will be monitored very closely every day to be sure to notice any negative problems that might arise. If any signs mentioned before are signalled the animal will be taken out of the experiment and will be euthanized.

Indicate the likely incidence.

We expect that minimal 98% of the animals will experience mild discomfort, because we will not perform harmful procedures or expect an adverse effect of the bacterial extract. This means that <2% of the animals might have unexpected moderate or severe discomfort due to for example stress or sickness.

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).

According to the EU-guidelines 98% of the animals the cumulative discomfort will be moderate because of the the combination of all the procedures planned. This includes the discomfort of individual housing for the BALB/cJ and their control group and the longitudinal oral gavage and testing. For a maximum of 2% of the animals the level of discomfort can rise up to severe discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

At the end of the experiment we will determine ex vivo changes for which we need the brains of the mice to assess the mechanisms by which OM-85

lead to possible structural and functional changes in specific brain regions. Even so, the brain and a part of the intestine/caecum will be further researched after euthanizing the animal.

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

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

[X] Yes

5.

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer 2015-0131
2. Titel van het project: From belly to brain: the role of gut bacteria in brain and behavior using mouse models of autism and attention disorders.
3. Titel van de NTS: Van buik naar brein: de rol van darmbacteriën in autisme en attentie-stoornissen.
4. Type aanvraag:
   - ☐ nieuwe aanvraag projectvergunning
5. Contactgegevens DEC:
   - Naam DEC: RUDEC
   - Telefoonnummer contactpersoon: [redacted], bereikbaar op maandag, dinsdag, en donderdag van 9:00 tot 15:00 uur
   - Mailadres contactpersoon: [redacted]
6. Adviestraject:
   - ☐ ontvangen door DEC: 19-01-2016
   - ☐ aanvraag compleet
   - ☐ in vergadering besproken: 02-02-2016
   - ☐ anderszins behandeld
   - ☐ termijnonderbreking(en) van 08-02-2016 tot 16-02-2016
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   - ☐ aanpassing aanvraag: 16-02-2016
   - ☐ advies aan CCD: 17-03-2016
7. Eventueel horen van aanvrager
   - Datum
   - Plaats
   - Aantal aanwezige DEC-leden
   - Aanwezige (namens) aanvrager
   - Strekking van de vraag / vragen
   - Strekking van het (de) antwoord(en)
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag
8. Correspondentie met de aanvrager
   - Datum: 08-02-2016
   - Strekking van de vragen:

     Niet-technische samenvatting:

     - 3.2 De verwachting over het vinden van biomarkers en de behoefte daaraan, is niet uitgewerkt in het project. De onderzoekers worden verzocht dit in overeenstemming met elkaar te brengen.

     -3.4 De commissie adviseert de onderzoekers de naam van het bedrijf te verwijderen.

     -3.1 Uit de beschrijving in het projectvoorstel blijkt dat het om een bacterieel extract gaat. Dit wordt hier niet duidelijk vermeld.

-3.2 Dit is niet de eerste studie over microbioom en brein. De onderzoekers worden verzocht dit anders te formuleren.
-3.3 De eerste zin volstaat om dit onderdeel te beantwoorden. De overige informatie kan worden toegevoegd aan onderdeel 3.1
-3.5 Zie opmerking bij DAP-1 K. Voor een aanzienlijk deel van de 98% met licht ongerief zal deze inschatting te laag blijken.
-4.3 Het antwoord bevat niet de gevraagde informatie over de keuze voor de diersoort en waarom de gekozen diermodellen de meest verfijnde zijn voor het behalen van de doelstelling.
-4.4 De laatste zin behoeft enige nuancering: alle dieren zullen gewicht verliezen vanwege de voedselrestrictie of lijden vanwege de experimenten zonder dat zij uit de proef gehaald worden.

Project Proposal:
-3.1. De onderzoekers willen het effect van [redacted] onderzoeken. Kunnen de onderzoekers de aard en de kinetiek van deze stof wat uitgebreider toelichten? Beïnvloedt deze stof de darmflora gedurende langere tijd? De commissie begrijpt niet goed waarom u deze stof gedurende 10 dagen geeft en pas na drie rustdagen het microbioom en het gedrag zult testen (zie ook de vraag bij DAP1).
-3.1 Welke veranderingen in darmflora van mensen zijn gevonden na inname van [redacted] Is er in de mens al eerder gezocht naar afwijkingen in het microbioom van ASD en ADHD patiënten en zijn deze gevonden?
-3.1 De commissie neemt aan dat dit onderzoek gebaseerd is op de resultaten van eerder onderzoek met [redacted]. Zij mist echter een gedegen beschrijving van die resultaten, zoals de effecten die het heeft op de darm en het bloedbeeld. De onderzoekers worden verzocht deze beschrijving toe te voegen.
-3.3 De noodzaak voor het vinden van biomarkers, en de wijze waarop het project zal kunnen leiden tot identificatie hiervan, lijkt hier secundair en is onvoldoende toegelicht.
-3.4.1. De onderzoekers willen drie modellen voor ADHD en autisme gebruiken. Kunnen zij duidelijker uitleggen waarom de eerste twee modellen (VPA en DAT -/-) niet volstaan, waardoor het derde model (BALB/cJ) ook nodig is?
-3.4.2. De onderzoekers zullen geen vervolgexperimenten doen wanneer zij in een model geen verschil zien in microbioom en/of wanneer [redacted] toediening geen effect heeft. Bedoelen zij hiermee een effect op het microbioom of op het gedrag? De commissie is van mening dat ook het uitblijven van een effect op het gedrag een reden is om af te zien van verdere experimenten in het betreffende model.
-3.4.2. In het tweede deel van het onderzoek willen de onderzoekers MRI scans van de muizenhersenen maken. Welke vraagstelling willen de onderzoekers beantwoorden met deze MRI scans? Dit is nog onvoldoende toegelicht in de achtergrondbeschrijving (onderdeel 3.1).

Description of Animal Procedures:
DAP1
-A1. De gebruikte dosis is op grond van de gegeven informatie niet te beoordelen. Waarom willen de onderzoekers een rustperiode van [redacted] inlassen nadat de stof 10 dagen is

geven? Als de onderzoekers verwachten dat de stof een langdurig/chronisch effect heeft dat nog te meten is drie dagen na de laatste dosis, waarom willen zij deze stof dan gedurende 10 dagen geven?
-A1. Onderdeel 3. Willen de onderzoekers bepaalde clusters van bacteriën in het microbioom onderzoeken, en zo ja welke? Gaan zij brede sequencing van het microbioom doen? Welke assay gaan zij hiervoor gebruiken, of aan wie gaan zij dit uitbesteden?
-B: De C57Bl/6 van Jackson is de negatieve controle voor de DAT -/- muis. Voor het VPA muismodel worden C57Bl/6 muizen van Charles River gebruikt. Zijn de muizen van Jackson daar niet geschikt voor? Dit zou een flink aantal muizen schelen omdat één controlegroep voor zowel de DAT -/- muizen als het VPA muismodel gebruikt kan worden. De onderzoekers worden verzocht hun huidige keuze beter toe te lichten of het aantal dieren hier en in DAP2 aan te passen.
-K: De onderzoekers gaan KO-muizen gebruiken. Wat is het ongerief van het fenotype? De BALB/cJ en de controledieren worden gedurende langere tijd individueel gehuisvest. Volgens bijlage VIII van de EU-richtlijn geeft dit matig ongerief, maar dit is nergens vermeld. De commissie is van mening dat het cumulatief ongerief voor alle dieren door de combinatie van alle handelingen ingeschaald zou moeten worden als matig.
DAP2
- A1. Onderdeel 3. De onderzoekers zullen niet alleen de bacteriesamenstelling van de muizenkeutels bepalen, maar ook de lumen- en mucus-geassocieerde bacteriën in het caecum van de muizen. In hoeverre is dit relevant voor het microbioom van patiënten? Zijn deze resultaten wel vertaalbaar naar de mens?

- Datum antwoord: 16-02-2016
- Strekking van de antwoorden:
-

Niet-technische samenvatting:
-3.2 Op verzoek van de DEC commissie hebben we de verwachting over het vinden van biomarkers in overeenstemming proberen te brengen.
-3.4 De naam van het bedrijf is op verzoek van de DEC commissie niet meer aanwezig in dit onderdeel.
-3.1 In de NTS is op verzoek van de commissie de informatie toegevoegd die gevraagd werd.
-3.2 Anders verwoord.
-3.3 Wijzigingen doorgevoerd die werden voorgesteld door de commissie. De eerste zin is blijven staan terwijl de overige informatie is verplaatst naar het onderdeel 3.1 in de NTS.
-3.5 We zijn het eens met de DEC Commissie. Voor de dieren is het niveau van ongerief op matig gezet.
-4.3 De keuze van de diersoort en waarom de gekozen diermodellen de meest verfijnde zijn voor het behalen van de doelstelling is gewijzigd in het aangegeven onderdeel. Hier is nu vermeld dat we hebben gekozen voor verschillende muismodellen. De gekozen modellen hebben elk een andere oorsprong en etiologie van de stoornissen. Ook vertonen ze verschillende symptomen van Autisme, ADHD of een combinatie hiervan. Muizen hebben volgens onderzoek verder ook een redelijk vergelijkbaar fysiologische en anatomische structuur van de darm als de mens. Daarom overtreft de kennis van de muis gastro-enterologie, genetica en immunologie elk ander model. Dit maakt de muis de beste keuze in

deze studie en zijn de resultaten het beste vertaalbaar naar de mens. De keuze voor de precieze muismodellen is nu ook verder toegelicht met de informatie uit onderdeel 3.4.1 van het project proposal.
-4.4 Naar aanleiding van de opmerking van de DEC Commissie hebben we enige nuancering aangebracht in dit onderdeel. We zullen een criterium hanteren van 85% van het vrije voedsel gewicht. Indien de dieren hieronder zitten zullen ze uit de studie worden gehaald. Hetzelfde geldt voor het lijden van pijn. Indien er zichtbaar geleden wordt door de dieren zal in overeenstemming met een dierverzorger gekeken worden naar de beste oplossing voor verlichting. Dit kan betekenen dat er pijnstillers worden gegeven of dat de muis uit de studie wordt gehaald en euthanasie zal plaatsvinden.

Project Proposal:
-3.1. Deze informatie is deels opgenomen in onderdeel 3.1 en deels in de extra informatie bijgevoegd als bestand bij ‘notes’ aan de aanvraag. [redacted]

[redacted]

Het belangrijkste voor het design van ons onderzoek is dat het geneesmiddel veel bacteriële bestanddelen bevat die werkzaam zijn op het immuunsysteem en zijn actief via de darmen. Een langdurig effect op de aangeboren immuunresponse in de darm van de gekozen doses [redacted] zijn bij het leverende bedrijf gevonden in dieren. Voor het design qua tijd hebben we gekozen voor de tijdsduur die eerder gebruikt is in muizen en die te vertalen is naar de behandeling in mensen. Dit leidde tot de huidige opzet voor de studie voor onze muizen waarbij is gekozen voor een design van 10 tot 30 dagen. Om er zeker van te zijn dat alle mogelijke veranderingen in het microbioom hebben plaatsgevonden en de stress te verminderen wordt er na de laatste dosis van het bacteriële extract een rust ingelast van enkele dagen. Deze informatie is nu te vinden in DAP 1.

-3.1 De veranderingen in de darmflora van mensen na inname van [redacted] waar de DEC Commissie naar vraagt zijn nog onbekend. Tot nu toe zijn er geen studies gedaan naar het effect van het bacteriële extract op de darmen in klinisch onderzoek. Op dit moment is er onderzoek gaande naar het effect van [redacted] op de samenstelling van bacteriën in de neusholte in kinderen van 6 maanden tot 2 jaar. Dit [redacted] onderzoek is nog niet afgerond en de resultaten zijn daarom ook nog niet geanalyseerd. Hetzelfde geldt voor recent klinisch en niet-klinisch werk dat wordt uitgevoerd in astma en moeilijke ademhaling (wheezing) in kinderen en de vergelijking daarvan met data van muis studies. Over beide projecten is daarom geen informatie opgenomen in het project.
Over de microbioom afwijkingen in patiënten met autisme spectrum stoornis, waar de commissie naar vraagt, is er bekend dat er verschillen zijn met controle proefpersonen. In klinische observaties is gezien dat de darmen en factoren in het dieet gedragssymptomen kunnen verslechteren dan wel verbeteren in een subgroep van personen met autisme spectrum stoornis (ASD)(MacFabe, 2015; Frye, 2015; Cryan, 2012). In zijn review uit 2015 schrijft MacFabe dat uit onderzoek blijkt dat SCFA stofwisselingsproducten van het darmmicrobioom opmerkelijke effecten hebben op de fysiologie van de patiënt zoals de hersenen en het gedrag. Door het gebruik van een translationeel diermodel, in vitro en klinische studies blijkt dat SCFA's unieke eigenschappen hebben, met name met neuroplasticiteit, geheugenfunctie, GPCRs, darm fysiologie, weefselbarrière permeabiliteit, oxidatieve stress, mitochondriale functie, carnitine metabolisme en epigenetica, die zijn betrokken bij ASD. Verder zijn er veel potentiële klinische scenario's voor genetische milieu-interacties, die consistent zijn met verhoogde blootstelling aan of verminderd metabolisme van SCFA's in een individu met een risico op ASD.
In Attentie-deficit/ hyperactiviteit disorder (ADHD) is minder onderzoek gedaan. ADHD is een neurologische aandoening geassocieerd met afwijkingen in dopamine neurotransmissie en tekorten in de verwerking van beloning. Het microbioom zou kunnen bijdragen aan ADHD etiologie via de darm-hersen-as volgens recent onderzoek in Nijmegen (data onder review). De onderzoekers hebben onderzocht of er verschillen in het microbioom zijn tussen ADHD gediagnosticeerde patiënten en controles. Ook werd het verband hiervan onderzocht met de neurale beloningsverwerking. Uit de resultaten blijkt dat de relatieve hoeveelheden van verschillende bacteriële taxa verschillen tussen patiënten en controles. Ze vonden een nominale toename van het genus Bifidobacterium bij ADHD patiënten. Deze stijging is gekoppeld aan een verbeterde functionaliteit van het bacteriële gen dat codeert voor cyclohexadienyl dehydratase. Dit enzym is betrokken bij de synthese van fenylalanine, een voorloper van dopamine en wordt geassocieerd met een verminderde fMRI reactie in de ventrale striatum tijdens beloninganticipatie. Deze resultaten suggereren dat de verschillen in het microbioom voorspelde functie bestaat tussen ADHD patiënten en controles (data onder review).
-3.1 Op verzoek van de DEC commissie is een uitgebreidere achtergrond van [redacted] bij de eerste vraag/opmerking uitgewerkt. Dit is terug te vinden in de background (onderdeel 3.1) en in de extra informatie die additioneel is bijgevoegd aan de projectaanvraag onder ‘notes’.
-3.3 De reden voor het zoeken van biomarkers is gebaseerd op de noodzaak van surrogaat uitkomstmaten die toegankelijk zijn (bijvoorbeeld verhouding van bacteriële samenstelling in de darm die gedragskenmerken voorspellen) plus als basis voor target ontdekking (om vast

te stellen of wijzigingen in de darm microbioom een levensvatbare therapeutische benadering kan zijn als benadering van ASD en ADHD).
-3.4.1. De keuze voor een derde muismodel is vanwege dat het BALB/cJ model en hun controle model (BALB/cByJ), modellen zijn die voor zowel ASD als ADHD kan worden gebruikt. Zo modelleren ze veranderingen in sociaal gedrag, impulsiviteit-compulsivity en aandacht. Bij de mens zijn de aandoeningen vaak comorbide aanwezig, wat betekent dat deze patiënten zowel ASD en ADHD hebben. Dit gecombineerde fenotype is de basis voor de selectie van BALB/c model dat aspecten van ADHD en ASD eigenschappen vertoont. De reden voor het gebruik van de DAT - / - (KO) muis is, dat het gerapporteerd wordt als een "gouden standaard" ADHD model en daarmee veranderingen in het microbioom in dit model zou overtuigen in het onderzoeksveld. Hetzelfde geldt voor het valproaat (VPA) geïnduceerde model (VPA). Dit wordt ook gezien als een 'gouden standaard' model voor de symptomen van ASD op zichzelf.
-3.4.2 We zijn het eens met de DEC. Als er geen veranderingen worden gevonden in het gedrag van de dieren, zal het tweede experiment niet worden uitgevoerd.
-3.4.2. In onderdeel 3.1 is meer opgenomen over het scannen van de muizen. Het zal informatie geven over mogelijke structurele [redacted] en functionele [redacted] connectiviteit veranderingen die optreden als gevolg van de [redacted]. Dit kan binnen en tussen de modellen en de controlegroepen. [redacted]
Onze onderzoeksvraag is of dit wordt teruggedraaid door langdurige microbioom interventie met [redacted] Even terzijde, worden in de literatuur over ASD momenteel verschillen in korte versus lange afstand connectiviteit als kern van het ontstaan van het fenotype vastgesteld. Dit project zal informatie toevoegen aan deze resultaten. Met betrekking tot de toestand van resting-state analyse: we hebben niet de hypothese dat frontostriatal default mode netwerken veranderbaar zijn door het microbioom, maar aangezien deze data-acquisitie relatief kort is willen we deze vraag beantwoorden in diezelfde dieren die we in vivo MRI-DTI studies door de scan uit te voeren.

Description of Animal Procedures:
DAP1
-A1. De doses zijn bepaald in primaire farmacologische modellen [redacted] behalve bij chronische modellen. Daarbij werd een verlengde toediening gebruikt. Het advies van het bedrijf die de productie van [redacted] doet, heeft de beslissing beïnvloed qua design van het onderzoek met rustpauzes aangezien ze stellen dat het extra tijd kost voor de [redacted] om het effect op het microbioom te maximaliseren na de laatste toediening. Dit heeft te maken met de 'turn-around' tijd van het bacteriële extract op de darmbacteriën.
Bovendien is het toedieningsschema aangepast aan het gedragsmodel vanwege dat een langere periode van toediening een negatieve invloed heeft op de stress en daarmee het lijden van de dieren door herhaalde toedieningen. Rustperioden geven daarvoor de mogelijkheid om niet alleen de darmbacteriën te laten opbloeien maar geven ook rust aan het dier.

-A1. Onderdeel 3. We zullen in de eerste plaats bacteriën onderzoeken die monoamine signalering beïnvloeden vanwege dat [redacted] Echter zullen we ook streven naar een bredere sequencing (Illumina-based 16S ribosomale RNA sequencing assays), die bij [redacted] uitgevoerd zullen worden. Hierbij wordt getracht te ontdekken of er andere bacteriële clusters dan [redacted] verband houden met deze fenotypen.
-B: We waarderen de opmerking van de DEC commissie dat het jammer is dat het aantal van C57BL/ 6 muizen die nodig zijn van twee bronnen (Jackson & Charles River) komen het aantal dieren verhoogd. Echter, door controledieren te gebruiken die afkomstig zijn van dezelfde leverancier, zorgen wij ervoor dat de milieu-invloeden op het gedrag en de muis microbiome hetzelfde zal zijn. Als we dit niet doen, lopen we het risico niet de juiste controle te hebben op het microbiome van C57BL/6 muizen. Dit omdat dit bij muizen van Charles River kan verschillen van die muizen die oorspronkelijk komen van Jackson. De DEC Commissie stelt terecht dat dit mogelijk zou moeten zijn door het gebruik van Jackson geproduceerde C57Bl/6 muizen als controles in deze experimenten. Maar dit heeft geen invloed op het eind aantal dieren dat nodig is. Dit vanwege dat de VPA controles sham-injecties zullen moeten krijgen met een placebo, terwijl de controles voor de DAT-KO geen injectie zouden moeten krijgen. We zijn blij om Jackson C57BL/6 controles op te nemen, maar dat zou dan enkel een kosten technische reden hebben.
-K: We zijn het eens met de DEC Commissie. Voor de dieren is het niveau van ongerief op matig gezet.

DAP2
- A1 Op basis van de onderstaande kennis hebben wij gekozen om ook naar de lumen- en mucus-geassocieerde bacteriën te kijken: Het distale deel van het darm microbioom wordt meestal onderzocht via de ontlasting vanwege de niet-invasieve wijze van inzameling. Het verschaft tegelijkertijd belangrijke inzichten in de distale microbioom samenstelling en functie. Het is echter belangrijk om te erkennen dat hechtende microbiota op de mucosale oppervlakken van de darm enorm kan verschillen van de aanwezige bacteriën in fecaal materiaal; daarnaast zitten er ook onverteerbare stoffen, dode darmwandcellen, galkleurstof, slijm en een kleine hoeveelheid water en zouten in de ontlasting. Bovenop de mogelijke verschillen met de keutels, kunnen verschillende compartimenten van de darm lokaal worden gekoloniseerd door verschillende gemeenschappen van bacteriën (Mulle, 2013).
Voor mensen is dit relevant omdat er is gebleken dat de darmwand van patienten met ASD abnormale communities hebben van verteringsbacteriën en daardoor vaker problemen hebben met hun verteringsstelsel. De vaker voorkomende ontstekingen zorgen ervoor dat de voedingsstoffen niet worden opgenomen zoals bij gezonde personen. Dit heeft ook effect op de mono-amines die veelal in de darmen worden geproduceerd in bacteriën in de mucus laag (Kelly et al. 2015; Kilian et al. 1998).
De vraag van de DEC commissie of de resultaten van de muizen vertaalbaar zijn kan beantwoord worden met het artikel van Nguyen et al. 2015. De onderzoekers stellen dat de muis en mens een redelijk vergelijkbare fysiologische en anatomische structuur hebben. Het verteringsstelsel in beide soorten is opgebouwd uit organen die anatomische gelijk zijn.

Verder is ook de gemiddelde ratio van darmoppervlakte en lichaamsoppervlakte gelijk tussen de muis en de mens (Nguyen, 2015). Tot nu toe zijn muismodellen het vaakst gebruikt in de microbiome onderzoeken. De kennis van de muis gastro-enterologie, genetica en immunologie overtreft daarom elk ander model. We beseffen dat er ook limitaties zijn aan het gebruik van de gekozen muismodellen, daarom zullen de uiteindelijke conclusies met de nodige voorzichtigheid gemaakt worden.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

9. Eventuele adviezen door experts (niet lid van de DEC)
   - Aard expertise
   - Deskundigheid expert
   - Datum verzoek
   - Strekking van het verzoek
   - Datum expert advies
   - Expert advies

B. Beoordeling (adviesvraag en behandeling)

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

C. Beoordeling (inhoud):

1. Het project is:
   - uit wetenschappelijk oogpunt verantwoord

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.
3. De DEC onderschrijft het belang van de doelstelling, namelijk 'to better understand the relationship between the gut microbiome and brain and behaviour in mice, in the context of neurodevelopmental disorders.' De te behalen onderzoeksresultaten zullen duidelijk maken of het darmmicrobioom in muismodellen voor autisme en ADHD anders is dan in normale (controle) muizen, en wat het effect is van de inname van een immuunmodulerend bacterie-extract op hersenen en gedrag in deze muismodellen. Voorts wordt duidelijk langs welke wegen dit effect wordt bereikt: neuraal, hormonaal of immunologisch. Deze resultaten kunnen op termijn bijdragen aan de ontwikkeling van nieuwe behandelingen voor deze aandoeningen. Ook kunnen mogelijk biomarkers voor autisme en ADHD worden vastgesteld. Beide aandoeningen komen regelmatig voor (prevalenties 2-5%), en hebben veel impact op het leven van getroffenen en hun naasten. De diagnose wordt doorgaans al op jonge leeftijd gesteld, waarna veel van deze kinderen langdurig met psychofarmaca worden behandeld. Maatschappelijk is dit onderzoek van belang, omdat de resultaten kunnen bijdragen aan een beter begrip van de etiologie en de ontwikkeling van nieuwe therapieën, wat niet slechts zou resulteren in gezondheidswinst voor veel mensen, maar ook zou kunnen leiden tot een minder ingrijpend alternatief voor de huidige behandeling met psychofarmaca die niet (door iedereen) als ideaal wordt beschouwd. De DEC

acht dit onderzoek daarom van substantieel belang.

4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Deze groep heeft veel ervaring in dit onderzoeksveld en met de voorgestelde dierproeven, en is partner in een EU-netwerk over dit onderwerp. De gekozen aanpak leidt tot betrouwbare uitspraken over het effect van de inname van het onderzochte bacterie-extract op het darmmicrobioom, op de hersenen en op het gedrag in muismodellen voor autisme, ADHD of een combinatie van beide aandoeningen, en geeft meer inzicht in de moleculaire mechanismen die hierbij betrokken zijn. Parallel aan de dierproeven zal een klinische studie worden uitgevoerd, waarbij de patiënten hetzelfde bacterie-extract innemen en dezelfde gedragstests zullen uitvoeren als de muizen. Door deze aanpak kunnen verkregen inzichten uit beide studies elkaar aanvullen ten behoeve van het design van toekomstige behandelstrategieën.
5. Er is geen sprake van bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren.
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Het ongerief wordt hoofdzakelijk bepaald door de orale gavages. De DEC schat het ongerief als gevolg van de dagelijkse orale gavage (maximaal 3x10 dagen met [redacted] rust ertussen), de milde voedselrestrictie, de gedragstesten, het imagen onder anesthesie en het doden in als licht. Het ongerief van de individuele huisvesting van de BALB/cJ en BALB/cByJ muizen schat de commissie in als matig. De DEC is van mening dat de combinatie van al deze factoren tot maximaal matig ongerief leidt. Het betreft een langlopend experiment (7 maanden) waardoor 2% van de muizen kan uitvallen vanwege het bereiken van een humaan eindpunt. Het ongerief voor deze dieren wordt ingeschat als matig tot ernstig. Het cumulatief ongerief voor de muizen in de beschreven vergunningaanvraag is dus juist ingeschat als matig voor 98% van de dieren en ernstig voor 2% van de dieren.
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.** De doelstelling van het project kan niet gerealiseerd worden zonder proefdieren of door gebruik van minder complexe diersoorten. Een psychiatrische aandoening waarbij zowel genetische oorzaken als omgevingsfactoren en sociale interacties een rol spelen kan niet goed bestudeerd worden zonder proefdiermodellen.
8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de **vermindering** van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat en proportioneel ten opzichte van de gekozen strategie en de looptijd. De DEC is het eens met het beschreven onderzoeksmodel en de onderbouwing van het aantal benodigde dieren. De onderzoekers zullen eerst de optimale dosis en behandelduur bepalen. Door de stapsgewijze aanpak waarin de resultaten uit eerdere proeven worden gebruikt voor het design van vervolgexperimenten en door het inbouwen van goed omschreven go/no go momenten wordt onnodig gebruik van proefdieren voorkomen. De DEC is van oordeel dat het project kan worden uitgevoerd met maximaal 1140 muizen.
9. Het project is in overeenstemming met de vereiste van de **verfijning** van dierproeven. De orale gavage zal eerst worden getraind, zodat de stress voor de dieren in het experiment zoveel mogelijk wordt beperkt. Dagelijkse controles van de dieren zorgen ervoor dat bij onverwacht optredend ongerief tijdig kan worden ingegrepen. Het bacterie-extract wordt bloksgewijs toegediend, waardoor er rustperiodes voor de dieren zijn waarin er geen orale gavages zijn. Het bloed van de dieren wordt pas na het doden verzameld. De DEC is ervan overtuigd dat de dierproeven zo humaan mogelijk worden uitgevoerd.

Er is geen sprake van belangwekkende milieueffecten.

10. De niet-technische samenvatting is een evenwichtige weergave van het project, zelfstandig leesbaar, beknopt en begrijpelijk geformuleerd.

D. Ethische afweging

Op basis van de onder C genoemde overwegingen komt de DEC tot de volgende ethische afweging.

Met dit onderzoek worden wetenschappelijke inzichten verworven in het effect van het innemen van een bacterie-extract op het darmmicrobioom, op de hersenen en op het gedrag in muismodellen voor autisme, ADHD of een combinatie van beide aandoeningen. Voorts wordt duidelijk welke moleculaire mechanismen zijn betrokken bij dit effect. Op termijn kunnen deze resultaten bijdragen aan de ontwikkeling van nieuwe therapieën voor mensen met autisme en/of ADHD. Het belang van meer inzicht in de etiologie en de ontwikkeling van nieuwe therapieën acht de DEC substantieel, gezien de prevalentie van autisme en/of ADHD en impact van deze aandoening op patiënten en hun naasten. De resultaten uit dit onderzoek kunnen op korte termijn vertaald worden naar klinische toepassingen.

Tegenover dit substantiële belang staat het gegeven dat 98% van de dieren matig ongerief zal ondervinden en maximaal 2% ernstig ongerief zal ondervinden als gevolg van de orale gavages in combinatie met de benodigde handelingen. De commissie is er van overtuigd dat bij de dierproeven adequaat invulling gegeven zal worden aan de vereisten op het gebied van de vervanging, vermindering en/of verfijning van dierproeven. Het gebruik van de dieren en het daarbij optredende ongerief is onvermijdelijk, wil men de doelstellingen kunnen realiseren.

De DEC is van oordeel dat het hier boven geschetste belang de onvermijdelijke nadelige gevolgen van dit onderzoek voor de dieren, in de vorm van angst, pijn of stress, rechtvaardigt. Aan de eis dat het belang van de experimenten op dient te wegen tegen het ongerief dat de dieren wordt berokkend, is voldaan.

E. Advies

1. Advies aan de CCD

- De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
  - Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.

2. Het uitgebrachte advies is gebaseerd op consensus.

6.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD103002016489

Bijlagen
2

Datum 21 maart 2016
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 21 maart 2016.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002016489. 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).

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	10300
Naam instelling of organisatie:	Stichting Katholieke Universiteit Nijmegen
KvK-nummer:	41055629
Straat en huisnummer:	Geert Grooteplein 10
Postbus:	9101, t.a.v. [redacted]
Postcode en plaats:	6500 HB NIJMEGEN
IBAN:	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer:	UMC St Radboud

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

Gegevens plaatsvervangende verantwoordelijke onderzoeker

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

Gegevens verantwoordelijke uitvoering proces

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

Gegevens gemachtigde

BSN:	[redacted]
Naam:	[redacted]
Postbus:	9101
Postcode en plaats:	6500 HB [redacted] NIJMEGEN NIJMEGEN
Wilt u een nieuwe machtiging afgeven?	Ja
Wat mag de gemachtigde doen?	[ ] Een projectvergunning aanvragen [ ] Een wijziging op een verleende projectvergunning aanvragen [ ] Een melding doorgeven op een verleende projectvergunning [ ] Een bezwaarschrift indienen en daarover communiceren met de Centrale Commissie Dierproeven en alle andere handelingen verrichten die nodig zijn voor een goede afwikkeling van het bezwaarschrift [x] Alle bovenstaande opties

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:	21 april 2016
Geplande einddatum:	21 april 2021
Titel project:	From belly to brain: the role of gut bacteria in brain and behaviour using mouse models
Titel niet-technische samenvatting:	Van buik naar brein: de rol van darm-bacteriën in autisme en attentie-stoornissen
Naam DEC:	RU DEC
Postadres DEC:	Postbus 9101, 6500 HB Nijmegen (HP 231)
E-mailadres DEC:	dierexperimentencommissie@radboudumc.nl

Betaalgegevens

De leges bedragen:	€ 1.187,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

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

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Nijmegen
Datum:	21 maart 2016

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD103002016489
Bijlagen
2

Datum 21 maart 2016
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 21 maart 2016
Vervaldatum: 20 april 2016
Factuurnummer: 16700489
Ordernummer: Kostenplaats en kostensoort: 040823-461220: CDL projectnummer:2015-0131: Verantwoordelijk onderzoeker: [redacted]

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD103002016489	€ 1.187,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL41RBOS 056.999.6317 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD103002016489
Bijlagen
1

Datum 17 mei 2016
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 21 maart 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "From belly to brain: the role of gut bacteria in brain and behaviour using mouse models" met aanvraagnummer AVD103002016489. Wij hebben uw aanvraag beoordeeld.

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. Omdat het exact protocol en parameters niet voorafgaand het begin van het project bekend zijn, stelt de CCD de specifieke voorwaarde. De algemene voorwaarde betreffende artikel 10, lid 1 sub a van de wet wordt gesteld bij vergunningen met een langere looptijd. Dit om te voldoen aan datgene wat volgt uit dit artikel. U kunt met uw project "From belly to brain: the role of gut bacteria in brain and behaviour using mouse models" starten. De vergunning wordt afgegeven van 17 mei 2016 tot en met 21 april 2021. De looptijd van de vergunning wijkt af omdat de startdatum in de aanvraag in het verleden ligt.

Overige wettelijke bepalingen blijven van kracht.

Beoordeling achteraf
Na afloop van het project zal er een beoordeling plaatsvinden, zoals bedoeld in artikel 10a1, lid 1 sub d en lid 3, in de wet. Meer informatie over de eisen bij een beoordeling achteraf vindt u in de bijlage.
In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie RU DEC gevoegd. Dit advies is opgesteld op 17 maart 2016. 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.
Alg

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

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

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

deze projectvergunning voor het tijdvak 17 mei 2016 tot en met 21 april 2021, voor het project "From belly to brain: the role of gut bacteria in brain and behaviour using mouse models" met aanvraagnummer AVD103002016489, volgens advies van Dierexperimentencommissie RU DEC. In aanvulling op het advies van de DEC stelt de CCD een specifieke en een algemene voorwaarde.
De functie van de verantwoordelijk onderzoeker is [redacted] Voor de uitvoering van het project is Instantie voor Dierenwelzijn verantwoordelijk.

De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 21 maart 2016
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 21 maart 2016;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 21 maart 2016;
c Advies van dierexperimentencommissie d.d. 17 maart 2016, ontvangen op 18 maart 2016.

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Behaviour Pilot microbiome intervention	Muizen (Mus musculus) / BALB/cJ; BALB/cByJ; C57Bl/6 (VPA + controls); DAT-/-;	1020	2,00% Ernstig 98,00% Matig	
3.4.4.2 Behaviour in ASD and ADHD models with OM-85	Muizen (Mus musculus) / Gelijk aan dierproef 3.4.4.1.	120	2,00% Ernstig 98,00% Matig	

Voorwaarden
Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen
In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.
Deze beoordeling zal uiterlijk 21 april 2022 plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst de dierproeven conform de vergunning waren.

Specifieke voorwaarde:
De vergunning wordt verleend onder de voorwaarde dat het protocol voor de tweede studie vooraf wordt afgestemd met de Instantie voor Dierenwelzijn.

Algemene voorwaarde:
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 overleg met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarden wijzigen of intrekken.

Weergave wet- en regelgeving

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

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

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

niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

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

Beoordeling achteraf
Volgens artikel 10a1, lid 1d en lid 3 van de wet worden projecten waarbij niet-menselijke primaten worden gebruikt, projecten die als ernstig ingedeelde dierproeven omvatten of een dierproef die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, achteraf beoordeeld worden.