	Inventaris Wob-verzoek W16-04s								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 20151208								
1	Aanvraagformulier				x		x	x	
2	Brief mbt factuurinformatie				x		x	x	
3	Projectvoorstel oud				x		x	x	
4	Niet-technische samenvatting oud			x					
5	Bijlagen dierproeven oud			x					
6	DEC-advies				x		x	x	
7	Ontvangstbevestiging				x		x	x	
8	Brief CCD 17-08-2015				x		x	x	
9	Brief reactie 19-08-2015				x		x	x	
10	Projectvoorstel nieuw				x		x	x	
11	Niet-technische samenvatting nieuw	x							
12	Bijlagen dierproeven nieuw			x					
13	Advies CCD		x						x
14	Beschikking en vergunning				x		x	x	

1.

14 AUG 2015

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☑ Ja > *Vul uw deelnemernummer in* 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-Noord 10
Postbus	9101
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	[redacted] ☐ Dhr. ☑ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	

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

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

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	[redacted] ☐ Dhr. ☐ Mw.
		Functie	Instantievoor Dierenwelzijn
		Afdeling	[redacted]
		Telefoonnummer	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☑ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☐ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	0 8 - 0 9 - 2 0 1 5
		Einddatum	0 8 - 0 9 - 2 0 1 9
3.2	Wat is de titel van het project?	Elucidating the link between environmental factors and mitochondrial dysfunction leading	
3.3	Wat is de titel van de niet-technische samenvatting?	Effecten van de darmmicrobiota op ontwikkelingsstoornissen	
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 € 741,00 Lege

☐ Wijziging € Lege

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

☐ Via een eenmalige incasso

☑ 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, document factuurgegevens

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	Instantie voor dierenwelzijn
Plaats	Nijmegen
Datum	0 8 - 0 8 - 2 0 1 5
Handtekening	[redacted]

Radboud universitair medisch centrum
Concernstaf, sectie Kwaliteit en Veiligheid

Postbus 9101, 6500 HB Nijmegen
Huispost 628
Geert Grooteplein 10

[redacted]
www.radboudumc.nl

KvK 41055629/4

Geert Grooteplein 10
Postbus 9101
6500 HB Nijmegen

Datum
8 augustus 2015

Instantie voor Dierenwelzijn

Onderwerp
Factuurinformatie Projectaanvraag

Geachte CCD,

Hierbij sturen wij u de administratieve gegevens behorend bij de ingediende projectaanvraag. Wij verzoeken u de factuur te versturen naar de IvD als gemachtigde van de vergunninghouder. Hiervoor AUB het bij u bekend e-mailadres gebruiken ([redacted]).

Om verwerking door de financiële afdeling mogelijk te maken verzoeken wij u tevens ***op de factuur*** de volgende gegevens te vermelden:

Factuuradres:	Radboudumc 28 F&A crediteuren Postbus 9101 6500HB, Nijmegen
Kostenplaats en kostensoort:	040823-461220
CDL projectnummer:	2015-0077
Verantwoordelijk onderzoeker:	[redacted]

Bij voorbaat dank.

Met vriendelijke groeten

[redacted]
Instantie voor Dierenwelzijn
[redacted]

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.zbo-ccd.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.	Elucidating the link between gut dysbiosis and mitochondrial dysfunction leading to neurodevelopmental disorders

2 Categories

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

[] Basic Research
[X] Translational or applied research
[] Regulatory use of 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.

Neurodevelopmental disorders are disabilities associated mainly with the functioning of the neurological system and brain. Individuals with these disorders can experience difficulties with language and speech, behaviour, learning, motor skills, and other neurological functions. Most neurodevelopmental disorders are caused by genetic abnormalities, including fragile-X syndrome and Down syndrome. Other neurodevelopmental disorders are referred to as complex because they have multiple and complex contributors rather than one clear cause. These complex disorders typically involve cognitive, behavioural or personality characteristics (Tager-Flusberg, 1999a). Complex neurodevelopmental disorders, such as attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD), are common and affect both children and adults.
Development of the nervous system is a complex process involving differentiation of neurons from neural stem cells. These differentiating neurons require high levels of energy, generated by mitochondria in the form of adenosine triphosphate (ATP). Mitochondria are localised in synapses, and synaptic function can be disturbed by mitochondrial morphology, function, and alterations in amount of mitochondria per cell (Kageyama and Wong-Riley 1982). Mitochondrial dysfunction contributes to several neurodevelopmental diseases (Anitha, Nakamura et al. 2013). Prevalence of several co-morbid features, like learning disabilities, motor delay, developmental regression, seizures and gastrointestinal (GI) dysfunctions, is typically higher in people with both a neurodevelopmental disorder and mitochondrial dysfunction (Rossignol and Frye 2012; Hsiao, McBride et al. 2013). Induced mitochondrial dysfunction in rats led to certain behavioural, metabolic and brain changes consistent with several neurodevelopmental disorders. These changes include repetitive behaviours, hyperactivity, increased amounts of reactive oxidative stress (ROS), reduced levels of antioxidants, and microglial activation (Rodríguez-capote et al. 2008).
GI dysfunction, such as chronic diarrhoea, constipation or intestinal infection, is a co-morbidity of special interest given its high prevalence and high correlation with symptom severity in several neurodevelopmental disorders (Adams, Johansen et al. 2011). The mechanisms leading to these GI problems remain unclear. One of the explanations of these GI dysfunctions found in people with neurodevelopmental disorders is dysbiosis, a significant change in gut bacterial composition. Gut bacteria contribute to neurodevelopment and function (Cryan and Dinan 2012) and there is a growing number of studies reporting dysbiosis in individuals diagnosed with neurodevelopmental disorders (Finegold, Downes et al. 2012; Gondalia, Palombo et al. 2012; Williams, Hornig et al. 2012; Kang, Park et al. 2013; Borre, O'Keeffe et al. 2014).

The adult human (and mouse) gut microbiota is dominated by the bacterial phyla Firmicutes and Bacteriodetes and seems to be stable and resilient against short-term changes (Faith, Guruge et al. 2013). The infant gut microbiota on the other hand is less stable and stabilises when the infant is 2-3 years old. The infant gut microbiota can be influenced by multiple factors, including antibiotics administered to the infant or mother, level of breastfeeding, mode of delivery, and genetics (Fallani, Amarri et al. 2011). Bergström et al. studied the gut microbiota of infants in a three-year Danish study with a cohort of 330 infants. Infants between 9 and 18 months old showed a significant shift in gut microbiota with the change from breastfeeding to solid foods (Bergstrom, Skov et al. 2014). Once established, the gut microbiota can be altered by antibiotic treatment, lifestyle, long-term change in diet, and bacterial infections (De La Cochetiere, Durand et al. 2005; Dethlefsen, Huse et al. 2008; Marques, Wall et al. 2015).
We hypothesise a link between gut microbiota and neurodevelopmental disorders via mitochondria affecting behaviour and cognition. Bacteria can affect mitochondria in several ways, for example through short-chain fatty acids (SCFAs). SCFAs are also known to affect mitochondrial function (Belzacq, Haouzi et al. 2002, Hecker, Sommer et al. 2015), for example by inducing apoptosis in colonic epithelium cells. Some bacteria are able to modulate mitochondrial function in order to maintain their living environment by preventing host cell apoptosis or to promote bacterial spread by inducing apoptosis (Matarrese, Falzano et al. 2007; Stavru, Bouillaud et al. 2011). Distressed mitochondria generate signalling molecules such as mitokines. These mitokines exit the host cell and can bind to and regulate receptors present on all eukaryotic cells. Other bacterial species, for example Pseudomonas spp. are capable of disrupting mitochondrial surveillance in Caenorhabditis elegans. Mitochondria are responsible for the synthesis of haeme and iron-sulphur clusters. Mitochondria are an attractive target for bacteria because iron is essential for bacterial processes like DNA replication and metabolism. Therefore, bacteria developed several strategies, for example production of siderophores, to acquire iron from mitochondria. Disabling the mitochondrial surveillance pathway renders other virulence factors, anti-mitochondrial toxins or siderophores more effective (Liu, Samuel et al. 2014). Pseudomonas spp. are psychrotrophic bacteria, which thrive at low temperatures (0-4 degrees Celsius). These psychrotrophic bacteria are commonly found in dairy products. We hypothesise that people ingest these bacteria more often as people tend to keep dairy products in the refrigerater more often and for longer periods of time. Finally, bacteria can affect host health and neurodevelopment through short-chain fatty acids. These SCFAs (butyrate, acetate and propionate), produced mainly by gut bacteria, are absorbed by the intestinal epithelium. SCFAs are processed by the citric acid cycle in mitochondria and used in several processes. Butyrate is used by colonocytes as source of energy. Acetate and propionate are transported via the bloodstream to other tissues and organs. Acetate is used for the synthesis of long-chain fatty acids (Christ, 1968). Propionate can be used as substrate for gluconeogenesis, a process starting in the mitochondria. Oral administration of prionate to rats led to cognitive deficits, decreased social interactions, repititive behaviour and abnormal motor activity (Shutlz *et al*., 2008). Thus, SCFAs are processed by mitochondria and fulfill several functions in the host. Dysbiosis can lead to altered levels of short-chain fatty acids (SCFA).
A major cause of gut dysbiosis is treatment with antimicrobials. Various antibiotics are potential risk factors for neurodevelopmental disorders (Atladóttir, Henriksen et al. 2012; Desbonnet, Clarke et al. 2015; Rosenfeld 2015). In addition, several antibiotic classes administered, like fluoroquinolones or aminoglycosides, are associated with mitochondrial dysfunction as a result of the close similarities between mitochondria and the targeted bacteria. This is seen as a mild side-effect and is well tolerated by most treated individuals. However, this side-effect combined with the effect of antimicrobials on the gut microbiota could possibly influence neurodevelopment, and thereby cognition and behaviour in adult life.
Another major cause of dysbiosis is diet, a key factor in determining gut microbial composition. Certain diets are able to impact cognition and behaviour to a great extent. Dietary therapies have been attempted to treat or ameliorate symptoms of a wide variety of neurological disorders, such as autism, epilepsy and Parkinson disease. In addition, dietary supplementation, for example omega-3 fatty acid or vitamin supplementation, is reported to have positive effects on symptoms of ADHD (Bos, Oranje et al. 2015; Rucklidge, Frampton et al. 2014). Obesity during pregnancy is

associated with mitochondrial dysfunction in the offspring (Wu, Russell et al. 2015) potentially leading to neurodevelopmental disorders. Childhood obesity is associated with poorer academic achievements and greater decay of brain structure and function. Obesity is also associated with reduced gut microbial diversity (Ley, Backhëd et al. 2005). Turnbaugh et al. demonstrate that these changes in diversity affect the metabolic potential of the microbiota. They show that the microbiota from obese individuals is more efficient in harvesting energy from diet compared to the microbiota from lean individuals. This efficiency in calorie harvest is transmissible from humans to mice by colonising germ-free mice with human microbiota from obese or lean individuals, indicating that dysbiosis contributes to obesity (Turnbaugh, Ley et al 2006).
There is a growing body of research reporting significant effects of mitochondrial dysfunction on brain development and function. Studies reporting associations between dysbiosis and neurodevelopment are also numerous. However, most of these studies are correlational studies, studying the correlation rather than the causality. Dysbiosis affecting mitochondrial function potentially leading to neurodevelopmental disorders has not been studied to our knowledge.

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 objective of this project is to investigate the link between dysbiosis, mitochondrial dysfunction and developmental disorders. In order to study this link we have designed a research plan composed of multiple components. The first component of this project aims to investigate the contribution of gut microbiota or specific bacteria to behaviour and cognition. The second component of this project focusses on the link between gut dysbiosis and neurodevelopmental disorders. To study this we plan to study the effects of gut dysbiosis on mitochondrial function. With the third component of this project we intend to study effects of dietary or antimicrobial treatments on gut dysbiosis, cognition and behaviour. Finally, we plan to design antimicrobial or dietary interventions to normalise gut microbial composition resulting in healthy mitochondrial function and neurodevelopment.
Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with less distress to the mice. The central theme of our research group encompasses effects of diets on neuronal systems, emphasising cognitive disorders in relation with metabolism and cerebral circulation. Important tools available include neuroimaging, including MRS and DTI, histopathology, and behavioural test equipment.
The main objective should be achievable and realistic within the duration of the project because of the availability of knowledge, expertise and accommodation.
Important publications of our research group:

- [redacted] (2013)

[redacted]

3.3 Relevance

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

The prevalence of many complex neurodevelopmental disorders, like ADHD and autism spectrum disorder (ASD), has been increasing across recent decades. These disorders have a huge impact on the affected individual, family members and society. Co-occurring disorders are common, such as dyslexia, obsessive compulsive disorder, depression and eating disorders. Studies have reported ADHD symptoms in 30-50% of individuals diagnosed with ASD. Similarly, around 66% of persons with ADHD show features of ASD (Davis & Kollins, 2012). Progress in understanding these disorders has been slow and treatment options are limited. In this study we will investigate the link between dysbiosis and neurodevelopmental disorders.
We will study effects of certain diets and antibiotics, which are capable of causing gut dysbiosis, on brain development and function. Investigating interventions changing gut microbial compositions and thereby influencing behaviour and cognition will increase knowledge about developmental disorders and could potentially result in new therapeutic interventions to treat or ameliorate symptoms of neurodevelopmental disorders. In addition, we aim to elucidate the link between gut dysbiosis and neurodevelopmental disorders by studying the role of mitochondrial dysfunction in these disorders.

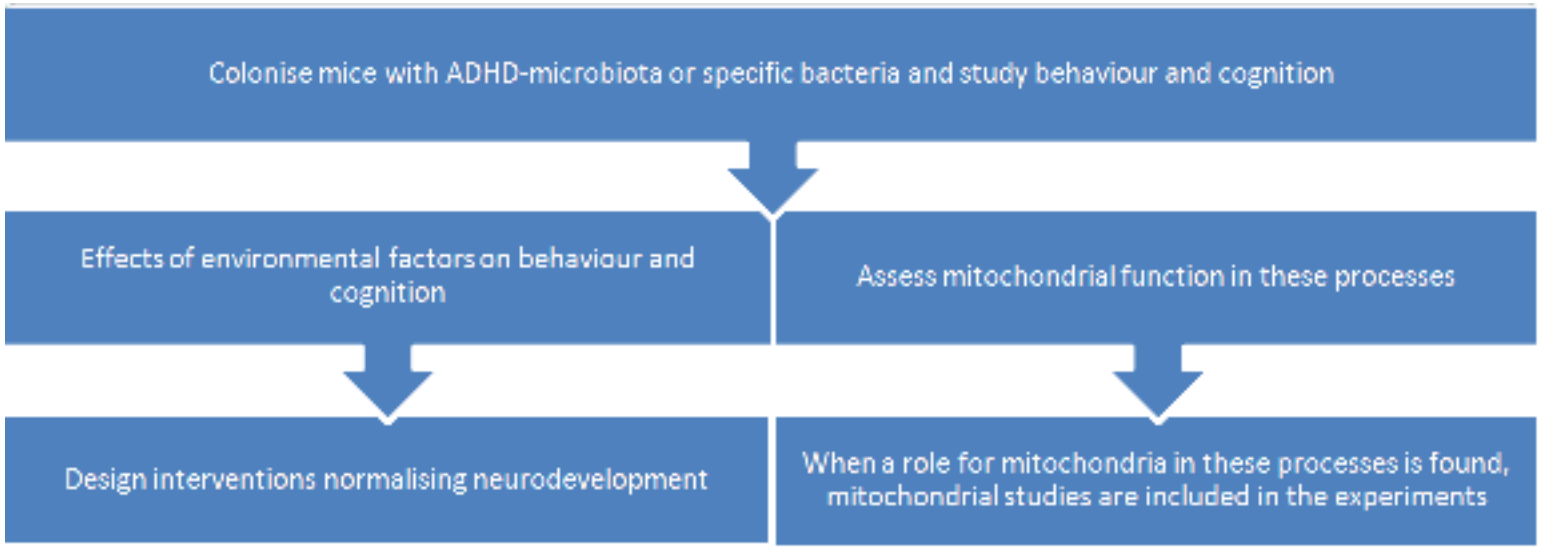
3.4 Research Strategy

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

Our main objective is to investigate the link between gut microbiota and neurodevelopment. In order to study this link we have designed several randomised experiments.

1. We aim to investigate the effects of dysbiosis on cognition and behaviour. We will do this by colonising mice with human microbiota from individuals with ADHD or by colonising mice with specific bacteria. After this we will study the impact of gut microbiota on behaviour and cognition.
2. We plan to study the link between gut dysbiosis, behaviour and function. Therefore, we will examine mitochondrial (dys)function after inducing dysbiosis.
3. We aim to study environmental factors that can lead to dysbiosis and study effects of dysbiosis on neurodevelopment.
4. We intend to design dietary and antimicrobial interventions to restore the gut microbiota to a healthy state, and thereby normalise neurodevelopment.

Overview of this project:



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.

1. Gut microbiota from people with ADHD is different in composition compared to gut microbiota from persons without ADHD (personal communication and Pärtty et al., 2015). First, we plan to study effects of the microbiota on cognition and behavior. Most gut bacteria cannot be cultured which makes studying their causal role in disorders technically challenging. To investigate whether gut dysbiosis is a cause or consequence of neurodevelopmental disorders, we will colonise germ-free mice with human microbiota from individuals with ADHD. Any dysbiosis present in the donor microbiota will be transferred to the recipient. Germ-free mice exhibit characteristics reminiscent of several neurodevelopmental disorders, like abnormal stress response and increased motor activity. This altered behaviour can be reversed by introducion gut microbiota. (Diaz Heijtz et al., 2011; Sudo et al., 2004). Thus, using conventionally raised mice, mice with normal gut microbiota, has the advantage that these mice don't show abnormal behavioural patterns and brain function. However, these mice already possess gut microbiota. When we introduce human gut microbiota in these mice, the 'new bacteria' will compete with the 'old bacteria'. Therefore, we prefer to use germ-free mice to study effects of gut microbiota op neurodevelopment. To study effects of the microbiota on neurodevelopment, we will investigate behaviour and cognition. ADHD in humans is associated with inattentiveness, hyperactivity, and/or impulsivity (American Psychiatric Association 2013). Anxiety is a very common co-morbidity in several neurodevelopmental disorders including ADHD. We can measure these characteristics in mice with behavioural tests, like the marble burying test and the open field test. Behavioural tests will be conducted to study changes in behaviour caused by gut microbiota. ADHD is not only characterised by behavioural changes but also by structural and functional brain differences (Weyandt, Swentosky et al. 2013). To cognition we will use neuroimaging

techniques, such as Diffusion Tensor Imaging (DTI), rs fMRI and Magnetic Resonance Spectroscopy (MRS), and histological and biochemical assays. After this we will compare microbial compositions from people with and without ADHD to identify suspect bacterial species for ADHD. In order to study behavioural and cognitive changes as result of specific bacteria we will colonise germ-free mice with specific bacterial species. These germ-free mice will be colonised only with these bacteria, enabling us to study behavioural and neuronal characteristics altered by these specific bacteria. After colonisation we want to examine cognition, behaviour and brain structure. When we see behavioural and/or cognitive changes as result of specific bacteria, we will infect conventionally raised mice with these bacterial species in order to mimic the natural situation.

2. Second, when we were able to induce dysbiosis by microbiota transplantation or by colonisation with specific bacteria, we plan to study the link between gut dysbiosis and neurodevelopment. To study this link we first have to induce gut dysbiosis. We will colonise mice with human ADHD-microbiota or with bacteria shown to be able to alter behaviour and cognition (first component). Inducing dysbiosis by diet or antibiotics is also possible, but inducing dysbiosis by colonising mice with microbiota or bacteria would be the best method as this will be almost instant, long-term, with only a few, mild side-effects. Changing gut microbial composition with diet takes a few months. Antibiotics directly affect mitochondria due to the striking similarities between bacteria and mitochondria, and has side-effects as well. After colonisation we will assess behaviour as well as mitochondrial function. We will also study effects of bacteria known to affect mitochondrial function, for example *Pseudomonas spp.* (Liu, Samuel et al. 2014; Manago, Becker-Flegler et al. 2015) on behaviour, cognition and mitochondrial function.

   When no altered mitochondrial function is found we will not measure mitocohndrial function in future experiments.

3. Third, we aim to investigate effects of environmental factors on gut microbiota and neurodevelopment. Hereby, we focus on two environmental factors: antibiotics and diet. These two factors are associated with dysbiosis, a significant change in microbial composition. In addition, we aim to study effects of certain diets on the composition of the gut microbiota. Certain long-term diets are able to change gut microbial composition and are capable of impacting cognition and behaviour to a great extend. We plan to expose mice to these environmental factors and consequently assess behaviour and cognition using various imaging, biochemical and physiological assays. The brain is a organ with high plasticity until the end of adolecence (around 3 months). In adulthood (3-6 months) the brain is fully grown, but remains plastic. We expect therefore, that we can still ameliorate symptoms of neurodevelopmental disorders later in life.
   If we were not able to induce gut dysbiosis in component 1 we will not continue.

4. If we were able to induce gut dysbiosis in component 1 , we will design specific intervention studies aiming at re-establishing a healthy microbiota. This should normalise mitochondrial energy production resulting in healthy neurodevelopment. Although diet can cause mitochondrial dysfunction via dysbiosis, these very same environmental factors, chosen carefully, could also normalise gut microbiota and

mitochondrial function promoting healthy neurodevelopment. The exact nature of these interventions will depend on the answers to the three above mentioned components of this project. We will first induce gut dysbiosis either by microbiota transplantation, or by introducing specific bacteria as this will be almost instant, long-term, with only a few, mild side-effects. After we see alterations in behaviour we will give the mice the selected diet. We will again conduct behaviour tests and thereafter we will measure cognition and mitochondrial function.

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

All components of this project are focussed on the link between dysbiosis and neurodevelopmental disorders such as autism and ADHD. We will address the effects of several interventions, such as diet or antimicrobials, on gut microbiota, mitochondrial function, and cognition and behaviour. These experiments will require critical timing as interventions and colonisations need to take place before finalisation of neurodevelopment.
To study this we formulated the following milestones:

1. Gut microbiota has an impact on neurodevelopment and we can identify suspect bacterial species affecting cognition and behaviour.
   If we observe affected neurodevelopment as result of the gut microbiota, we will explore the role of mitochondria in this process.
2. *Dysbiosis leads to mitochondrial dysfunction.*
   If we were able to demonstrate affected mitochondrial function in these processes, we will also assess mitochondrial function after dietary interventions. If we can show effects of gut microbiota and/or specific bacteria on behaviour and cognition, we will explore effects of diets or antibiotic treatment on gut microbial composition and neurodevelopment.
3. Environmental factors, such as diet and/or antibiotics, lead to gut dysbiosis and consequently affect neurodevelopment.
   When we observe effects of environmental factors on dysbiosis and on neurodevelopment, we will design dietary interventions to re-establish a healthy gut microbiota, leading to healthy neurodevelopment.
4. Mitochondrial energy production and neurodevelopment can be normalised by dietary interventions.

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	Effects of microbiota or specific bacteria on cognition and behaviour
2	Effects of antibiotics on gut microbiota and neurodevelopment
3	Effects of diet on gut microbiota and neurodevelopment
4	Interventions to re-establish a healthy gut microbiota

Format
Niet-technische samenvatting

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

1 Algemene gegevens

1.1	Titel van het project	Effecten van darmbacteriën op ontwikkelingsstoornissen zoals ADHD en autisme spectrum stoornis
1.2	Looptijd van het project	8-9-2015 - 8-9-2019
1.3	Trefwoorden (maximaal 5)	Ontwikkelingsstoornissen, antibiotica, voeding, darmbacteriën

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

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

3 Projectbeschrijving

3.1	Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)	Darmbacteriën vervullen belangrijke functies voor het menselijke lichaam. Zo spelen de darmbacteriën een essentiële rol in de werking van het centrale zenuwstelsel. Er zijn aanwijzingen dat verschillende ziektebeelden, waaronder ontwikkelingsstoornissen zoals autismespectrumstoornis (ASS) en ADHD, samengaan met verstoring van de samenstelling van darmbacteriën. Het is nog niet bekend of de verstoring in samenstelling van darmbacteriën oorzaak of gevolg is van de ontwikkelingsstoornissen. Wij willen graag onderzoeken of en hoe de darmbacteriën de breinontwikkeling beïnvloeden.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Complexe ontwikkelingsstoornissen, zoals autisme spectrum stoornis (ASS) en ADHD, worden veroorzaakt door meerdere, complexe factoren. Het aantal mensen dat gediagnosticeerd wordt met complexe ontwikkelingsstoornissen, is de laatste tientallen jaren enorm toegenomen. Er is weinig bekend over oorzaken van verschillende complexe ontwikkelingsstoornissen en op dit moment zijn behandelmethoden beperkt. Wij willen onderzoeken waardoor deze toename veroorzaakt wordt en hopen met deze kennis mogelijke therapeutische behandelingen tegen ontwikkelingsstoornissen te vinden.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Voor dit onderzoek zullen in totaal ongeveer 750 muizen gebruikt worden.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Gering ongerief als gevolg van wekelijkse gedragstesten en kortdurende orale toediening (gavage) van bacteriën. Behandeling met antibiotica veroorzaakt over het algemeen weinig bijwerkingen. Bijwerkingen die relatief vaak voorkomen, zoals diarree, geven vaak geringe, kortdurende, gevolgen voor het welzijn. Muizen kunnen mogelijk gering ongerief ondervinden als gevolg van dieet. Voor MRI-testen wordt algehele anesthesie toegepast waaruit de dieren niet zullen bijkomen.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Gering (90%), matig (10%)
3.6	Wat is de bestemming van de dieren na afloop?	Na afloop worden de dieren gedood en worden de organen voor verder onderzoek gebruikt.

4 Drie V’s

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Meerdere, vaak onbekende, factoren spelen een rol in de ontwikkeling van complexe ontwikkelingsstoornissen. Ook zijn gedrag en cognitie belangrijke parameters om ontwikkelingsstoornissen te onderzoeken. Hiervoor zijn nog geen proefdiervrije alternatieven beschikbaar.
4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	De muizen worden tevens gebruikt in diverse gedrags- en hersenfunctietesten. Er is uitgebreid gezocht in de literatuur of deze experimenten niet al eerder gedaan zijn waardoor deze experimenten overbodig zouden worden. Er zijn geen soortgelijke experimenten gevonden. De variatie tussen de muizen wordt zoveel mogelijk verkleind waardoor minder dieren nodig zijn voor significante verschillen. Als laatste is het minimale benodigde aantal muizen statistisch berekend aan de hand van eerdere soortgelijke studies.
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	In dit project zullen muizen gebruikt worden. Wij willen graag het effect van de darmbacteriën op gedrag en cognitie onderzoeken. Lagere diersoorten, zoals zebravissen, zijn hier niet voor geschikt. Over muizen is veel kennis beschikbaar en ook zijn veel studies naar de darmbacteriën uitgevoerd in muizen. Hiervan zal gebruik worden gemaakt tijdens dit project waardoor minder dieren nodig voor het optimaliseren van protocollen. Er is gekozen voor experimenten waarvoor zo weinig mogelijk muizen nodig zijn en dieren zo weinig mogelijk lijden. Mensen die deze experimenten uitvoeren zijn ervaren met proefdierwerk.
4.4	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	Dieren worden gezamenlijk gehuisvest in geschikte kooien om stress door eenzaamheid zoveel mogelijk te voorkomen. Ze zullen onbeperkt toegang hebben tot water en voedsel. Waar nodig zal algehele anesthesie gebruikt worden. Humane eindpunten, de eerste indicatie van ernstig lijden, zijn opgesteld om het leed van de dieren zo veel mogelijk te beperken. De muizen worden dagelijks gecontroleerd op welzijn en zo nodig wordt de dierenarts om advies gevraagd waarna dit advies wordt opgevolgd.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	

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.zbo-ccd.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 Effects of microbiota or specific bacteria on cognition and behaviour

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.

In order to study effects of the gut microbiota on neurodevelopment we plan to colonise mice with specific bacterial species or with human microbiota from individuals diagnosed with ADHD. After colonisation we aim to conduct behavioural tests followed by measuring mitochondrial function or performing neuroimaging tests. The general design for this experiment is as follows:

1. Colonisation

2. Behavioural tests

3. Measuring mitochondrial function or conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure post mortem brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.

All experiments, except neuroimaging and measuring mitochondrial function, will be performed in isolators wherein the mice are housed to prevent colonisation with environmental bacteria. As we house mice in isolators we are restricted in options for behavioural tests. This excludes all equipment requiring power. In addition, we cannot use equipment too large to fit inside the isolators (larger than 45x45cm).

We will colonise mice with microbiota or specific bacteria. We will collect stool samples once a week and sequence bacterial 16S rRNA genes. We will analyse stability of colonisation by comparing the taxonomic profiles of stool samples with the original sample.
Before and after colonisation we will conduct behavioural tests (at most once a week) in order to study behavioural changes after colonisation. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory.

After behavioural tests, mice are randomly divided into two groups: one group will be used for analysis of brain structure and function while the other group will be used to study post mortem brain mitochondrial function. Isoflurane, used to anaesthetise mice during neuroimaging, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.
Mice used for neuroimaging techniques are sacrificed directly after scanning by and their brains will be used to examine brain structure using histological and biochemical assays.

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

Before we start with this project, we will practise techniques, such as gavage, neuroimaging, transcardial perfusion, and measuring mitochondrial function, on surplus mice to optimise succes rate of these experiments.
This experiment will take maximally 4 weeks. The aim of this experiment is to study effects of the gut microbiota on neurodevelopment. We will first colonise mice with human microbiota or with specific bacteria to study whether gut dysbiosis is a cause of neurodevelopmental disorders. Any dysbiosis present in the donor microbiota will be transferred to the recipient mouse. Most of the bacteria present in gut microbiota cannot be cultured, this makes studying their causal role in disorders technically challenging. After colonisation we will conduct behavioural tests and analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders.
This approach allows us to study effects of gut microbiota on neurodevelopmental disorders. First, wel will colonise germ-free mice with human microbiota of specific bacteria. After that, we will study behaviour and cognition to investigate the causality between gut dysbiosis and neurodevelopmental disorders.
All mice will be colonised with a suspension of microbiota or specific bacteria in Phosphate Buffered Saline (PBS) via gavage. By oral force-feeding we can decrease variation as every mouse will receive exactly the same amount of microbiota. Administration of bacterial suspensions via gavage will have a mild, short-term impact on the animals. This procedure will be done once every week in order to reinforce the microbiota and will take about 5 seconds per mouse.
Behavioural tests can possibly cause mild, short-term distress. Tests conducted include the Open Field test, marble burying test, and object recognition test. These tests will be done at most once a week (before and after colonisation) in all mice and will take about 10 minutes each time. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory.
Brain function and structure, for example investigating cerebral blood flow, connectivity structures, or grey and white matter integrity, will be examined in one group of mice. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be sacrificed directly after these MRI experiments by transcardial perfusion-fixation using Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial

perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.Mitochondrial function will be measured in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed under comparable conditions. Introducing microbiota via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies.
We will first investigate if we are able to alter behaviour and cognition by microbiota transplantation or colonisation with bacteria. When we see behavioural and cognitive changes, we will include a group of mice to measure mitochondrial function. By practising challenging techniques on surplus animals, we will minimise the number of mice required for these experiments.

B. The animals

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

We will use germ-free as well as conventionally raised mice (Mus musculus). Germ-free mice, mice without microbiota, are required for colonisation. These mice have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice (3 weeks old), after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala.
Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females. Before we start this project we will practise the required skills with surplus mice. These skills include gavage, MRI scanning, transcardial perfusion, and measuring mitochondrial function. The maximum number of animals we consider to be necessary to practise is 40 mice in total in this project. We will use test groups (N=14), mice that receive human microbiota or specific bacteria, as well as control groups (N=14), mice receiving a sham treatment. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. The maximum number of animals we consider to be necessary is 224 mice, allowing us to conduct 8 experiments to analyse altered behaviour, brain function, and brain structure as result of microbiota or specific bacterial species. With these 8 experiments we plan to do the following procedures:

- 1 experiment to investigate whether ADHD gut microbiota affects behaviour and cognition in germ-free mice
- 4 experiments to analyse effects of specific bacterial species on behaviour and cognition in germ-free mice
  followed by:
- 3 eperiments to analyse effects of specific bacteria (selected in germ-free mice) species on behaviour and cognition in conventionally raised mice.

The specific bacterial species used will include Pseudomonas spp., often found in dairy products, and Lactobacillus and Bifidobacterium, often used in probiotics.
When behaviour and cognition are altered after colonisation, we will also include a group of mice to assess mitochondrial function (N=10).
Mitochondrial function will be assessed in mice colonised with the microbiota or bacteria shown to be most effective in changing behavioural and cognition. An additional 40 mice is required when mitochondrial function is proven to be affected by dysbiosis.
The total number of animals we consider necessary is 264 mice for this experiment and 40 mice to practise required skills.

Species	Origin	Maximum number of animals	Life stage
Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	304	3 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:
  We plan to investigate effects of gut microbiota or specific bacterial species on behaviour, cognition and mitochondrial function.
  The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.

- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. We will only assess mitochondrial function when we were able to affect behaviour and cognition by colonisation. Mitochondrial function is studied only for microbiota or bacteria shown to be most effective in changing behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. We will either colonise mice with microbiota or introduce specific bacteria. Using one of these methods, introduced gut dysbiosis will be almost instant, long-term and with least side-effects. Inducing dysbiosis with diet will take three months and using antibiotics to promote gut dysbiosis will also introduce side effects. After inducing intestinal dysbiosis we will conduct behavioural tests to assess behavioural changes due to the dysbiosis. The used behavioural tests give as little as possible distress and pain, while still yielding reliable results. When we see behavioural changes in the animals we will assess cognition in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

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

G. Location where the animals procedures are performed

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

[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, analgesiaor 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?

Negative effects from colonisation with bacteria, mostly gastrointestinal problems like diarrhoea, may be expected. Behavioural and cognitive changes caused by the colonisation such as hyperactivity and increased anxiety. We know from experience that distress caused by these changes is mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Effects of the microbiota or bacteria on behaviour and cognition are described in literature. Some of the bacteria are known to occasionally cause diarrhoea in humans. Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them. Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

Transplanting human microbiota to germ-free mice has been done before (Ridaura, Faith et al. 2013; Bruce-Keller, Salbaum et al. 2015) where the researchers studied the role of gut microbiota on obesity. No adverse effects, except a significant increase in weight, were reported. Our goal is to study effects of microbiota from individuals with ADHD on cognition and behaviour in mice. Distress caused by the expected behavioural and cognitive changes is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 1%.

K. Classification of severity of procedures

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

The discomfort caused by the colonisation can be classified as mild to moderate. Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category 'non-recovery'.
Cumulative levels of discomfort are expected to be mild (80%) to moderate (20%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed by cervical dislocation in order to study brain mitochondrial function and structure. The other group of mice will be sacrificed in order to study brain function and structure.

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

[] No > Descripe 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.zbo-ccd.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 Effects of antibiotics on gut microbiota and neurodevelopment

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.

In order to investigate effects of antibiotics on dysbiosis, mitochondrial function and brain development, we will expose mice to these antibiotic-treatments. Some antimicrobial classes are associated with gut dysbiosis and mitochondrial dysfunction. We will use often described antibiotics which possibly affect mitochondrial function, such as fluoroquinolones and aminoglycocides. In order to study effects of environmental effects on gut microbial composition, we will collect stool samples once a week.
After treatment we aim to conduct behavioural tests followed by measuring mitochondrial function or performing neuroimaging tests. The general design for this experiment is as follows:

1. Treatment of conventionally raised mice with antibiotics

2. Behavioural tests

3. Measuring mitochondrial function or conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure *ex vivo* brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.
Before and after treatment with antibiotics we will conduct behavioural tests in order to study behavioural changes after colonisation. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory.
After behavioural tests, mice are randomly divided into two groups: one group will be used for analysis of brain structure and function while the other group will be used to study brain mitochondrial function. Isoflurane, used to anaesthetise mice during neuroimaging, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.

Mice used for neuroimaging techniques are sacrificed directly after scanning and their brains will be used to examine brain structure using histological and biochemical assays.

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

This experiment will take maximally 5 weeks. The aim of this experiment is to study effects of antibiotics on gut dysbiosis and neurodevelopment. We will used conventionally raised mice, mice with normal gut microbiota. We will administer antibiotics via drinking water. We will select antibiotics known to affect mitochondrial function and often administered. To investigate effects of antimicribials on neurodevelopment, we will conduct behavioural tests and analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders.
This approach allows us to study effects of antibiotics on neurodevelopmental disorders. We will administer antibiotics to mice with normal gut microbiota. After that, we will study behaviour and cognition to investigate whether antibiotic-treatments affects neurodevelopment

All mice will be treated with antibiotics, such as fluoroquinolones, via drinking water. We will introduce these antimicrobial interventions to induce gut dysbiosis and after one month on antibiotic-treatment we will examine behaviour, brain structure, and brain function with behavioural studies and neuroimaging techniques. Every week (before and after treatment) stool samples will be collected to analyse microbial composition.
Behavioural tests, for example the Open Field test, the Marble Burying test, and the object recognition test, can possibly cause mild, short-term distress. These tests will be done at most once a week (before and after colonisation) in all mice and will take about 10 minutes each time. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory.
Brain function and structure, for example investigating cerebral blood flow, connectivity structures, or grey and white matter integrity, will be examined in one group of mice. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be sacrificed directly after these MRI experiments by transcardial perfusion-fixation using 1 M Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.
Mitochondrial function will be measured in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed in comparable conditions. Introducing microbiota via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. All experiments are performed by experienced researchers or technicians.
We will carefully select potential antibiotics in order to minimise the groups of mice needed for this experiment. Mitochondrial function will be measured only when we were able to show altered mitochondrial function in the previous experiment (animal procedure 1), or when we see behavioural and/or cognitive changes after antibiotica-treatment.

B. The animals

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

We will use conventionally raised mice (Mus musculus). These conventionally raised mice, mice with normal microbiota, have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice, after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala. Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females.
G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. We will use test groups (N=14), mice that receive antibiotics associated with mitochondrial dysfunction, as well as control groups (N=14). The maximum number of animals we consider to be necessary is 140 mice, allowing us to conduct 5 experiments to analyse altered behaviour, brain function, and brain structure as result of treatment with antibiotics. With these 5 experiments we plan to study effects of five selected classes of antibiotics, including aminoglycosides, beta-lactam, chloramphenicol, fluoroquinolones and oxazolidinones. We start with three of these classes that are most often administrered to young children or pregnant women. When we don't see effects of these three classes of antibiotics we will end this experiment.
When behaviour and cognition are altered after antibiotic-treatment, we will also use a group of mice to assess mitochondrial function (N=10). Mitochondrial function will be assessed in mice treated with antibiotics shown to be most effective in changing behavioural and cognition. An additional 40 mice is required when behavioural and cognition are proven to be affected by dysbiosis. The total number of animals we consider necessary is 180 mice for this experiment.

Species	Origin	Maximum number of animals	Life stage

Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	180	3 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:
  We plan to investigate effects of gut dysbiosis on behaviour, cognition and mitochondrial function. The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.

- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. We will only assess mitochondrial function when we were able to affect behaviour and cognition by antibiotics or when mitochondrial function is shown to be affection in animal procedure 1. Mitochondrial

function is studied only for antibiotics shown to be most effective in changing behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. We will administer mice antibiotics associated with affected neurodevelopment. The used behavioural tests give as little as possible distress and pain, while still yielding reliable results. When we see behavioural changes in the animals we will assess cognition in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane. Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

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

G. Location where the animals procedures are performed

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

[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, analgesiaor 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?

Mild side-effects from antibiotic treatment, mostly gastrointestinal problems like diarrhoea, may be expected. Behavioural and cognitive changes caused by the antibiotics such as hyperactivity and increased anxiety. We know from experience that distress caused by these changes is mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Antibacterials commonly cause some side-effects as antibiotics can destroy commensal bacteria living in the host. Our goal is to study effects of diet or usage of antibiotics on cognition and behaviour in mice. We expect to see behavioural and cognitive changes. Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them.Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

Occurrence of side-effects from antibiotics cannot be prevented. These potential effects are considered to be mild with no significant impairment of the well-being or general condition. Humane endpoints are adopted to minimise severity. Distress caused by the expected behavioural and cognitive changes is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 5%.

K. Classification of severity of procedures

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

The discomfort caused by the antibiotics can be classified as mild. Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category 'non-recovery'. Cumulative levels of discomfort are expected to be mild (100%).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed by cervical dislocation in order to study brain mitochondrial function and structure. The other group of mice will be sacrificed in order to study brain function and structure.

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

[] No > Descripe 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.zbo-ccd.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 3	Type of animal procedure Effects of diet on gut microbiota and neurodevelopment

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.

In order to investigate effects of diet on dysbiosis, mitochondrial function and brain development, we will expose mice to these dietary interventions. Certain diets are able to impact cognition and behaviour to a great extent. Diets selected in this experiment are associated with neurodevelopment, for example high fat or high sugar diets. In addition, we will also study effect of diets considered healthy like diets high in omega-3 or carbohydrates. In order to study effects of diet on gut microbial composition, we will collect stool samples once a week.
After treatment we aim to conduct behavioural tests followed by measuring mitochondrial function or performing neuroimaging tests. The general design for this experiment is as follows:

1. Diet conventionally raised mice

2. Behavioural tests

3. Measuring mitochondrial function or conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure *ex vivo* brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.

Before and after treatment with diets we will conduct behavioural tests in order to study behavioural changes after colonisation. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory.
After behavioural tests, mice are randomly divided into two groups: one group will be used for analysis of brain structure and function while the other group will be used to study brain mitochondrial function. Isoflurane, used to anaesthetise mice during neuroimaging, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.

Mice used for neuroimaging techniques are sacrificed directly after scanning and their brains will be used to examine brain structure using histological and biochemical assays.

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

This experiment will take maximally 3 months. The aim of this experiment is to study effects of diet on gut dysbiosis and neurodevelopment. We will used conventionally raised mice, mice with normal gut microbiota. We will give mice diets associated with neurodevelopment, for example high fat or high sugar diets. To investigate effects of diet on neurodevelopment, we will conduct behavioural tests and analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders.
This approach allows us to study effects of diet on neurodevelopmental disorders. We will give mice diet and after three months we will study behaviour and cognition to investigate whether diet affect neurodevelopment
All mice will be fed special diets, for example high saturated fat and high caloric/sucrose diets or diets with unsaturated fatty acids. We will introduce these dietary interventions to induce gut dysbiosis and after three months on the diets we will examine behaviour, brain structure, and brain function with behavioural studies and neuroimaging techniques. Every week (before and after treatment) stool samples will be collected to analyse microbial composition.
Behavioural tests can possibly cause mild, short-term distress. Tests conducted include the Open Field test, marble burying test, and object recognition test. These tests will be done at most once a week (before and after colonisation) in all mice and will take about 10 minutes each time. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory.
Brain function and structure, for example investigating cerebral blood flow, connectivity structures, or grey and white matter integrity, will be examined in one group of mice. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be sacrificed directly after these MRI experiments by transcardial perfusion-fixation using 1 M Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.Mitochondrial function will be measured in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed in comparable conditions. Introducing microbiota

via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. All experiments are performed by experienced researchers or technicians.
We will carefully select potential diets in order to minimise the groups of mice required for this experiment. Mitochondrial function will be measured only when we were able to show altered mitochondrial function in the previous experiment (animal procedure 1), or when we see behavioural and/or cognitive changes after diets.

B. The animals

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

We will use conventionally raised mice (Mus musculus). These conventionally raised mice, mice with normal microbiota, have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice, after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala. Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females.
G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. We will use test groups (N=14), mice that receive diets associated witn neurodevelopmental disorders, as well as control groups (N=14), mice given healthy diets. The maximum number of animals we consider to be necessary is 112 mice, allowing us to conduct 4 experiments to analyse altered behaviour, brain function, and brain structure as result of diet. With these four experiments we plan to study effects of four selected diets, including diets with high saturated or unsaturated fatty acids and high calorie/sugar diets.
When behaviour and cognition are altered after diet, we will also use a group of mice to assess mitochondrial function (N=10). Mitochondrial function will be assessed in mice given diets shown to be most effective in changing behavioural and cognition. An additional 40 mice is required when behavioural and cognition are proven to be affected by dysbiosis. The total number of animals we consider necessary is 152 mice for this experiment.

Species	Origin	Maximum number of animals	Life stage
Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	152	3 weeks

C. Re-use

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:
  We plan to investigate effects of gut dysbiosis on behaviour, cognition and mitochondrial function. The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.

- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. We will only assess mitochondrial function when we were able to affect behaviour and cognition by diet or when mitochondrial function is shown to be affection in animal procedure 1. Mitochondrial function is studied only for antibiotics shown to be most effective in changing behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. We will give mice diets associated with affected neurodevelopment. The used behavioural tests give as little as possible distress and pain, while still yielding reliable

results. When we see behavioural changes in the animals we will assess cognition in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

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

F. Accommodation and care

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, analgesiaor 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?

Some diets can cause mild distress. Mostly gastrointestinal problems like diarrhoea, may be expected. Behavioural and cognitive changes caused by the induced dysbiosis such as hyperactivity and increased anxiety. We know from experience that distress caused by these changes is mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Feeding mice modified diets, that do not meet all of the nutritional needs are expected to cause mild distress. Our goal is to study effects of diet on cognition and behaviour in mice. We expect to see behavioural and cognitive changes. Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them.Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

We will prefer to use diets that do meet all of the animals' nutritional needs to prevent distress. Potential effects from diets are considered to be mild with no significant impairment of the well-being or general condition within the time-scale of this study. Humane endpoints are adopted to minimise severity. Distress caused by the expected behavioural and cognitive changes is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 1%.

K. Classification of severity of procedures

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

The discomfort caused by the diets can be classified as mild. Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category 'non-recovery'.
Cumulative levels of discomfort are expected to be mild (100%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed by cervical dislocation in order to study brain mitochondrial function and structure. The other group of mice will be sacrificed in order to study brain function and structure.

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

[] No > Descripe 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.zbo-ccd.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 4	Type of animal procedure Interventions to re-establish a healthy gut microbiota

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.

We will carry out this experiment only when we see changes in behaviour and cognition after introducing antibiotics, diet or gut microbiota from ADHD individuals (previous describes animal procedures). The exact nature of this experiment largely depends on outcomes of previous experiments.
The brain is an organ with high plasticity. Therefore we plan to design dietary intervention studies to re-establish a healthy gut microbiota, thereby normalising mitochondrial energy production resulting in healthy neurodevelopment. We aim to ameliorate certain characteristics of developmental disorders, such as anxiety and abnormal motor activity. The general design for this experiment is as follows:

1. Introducing intestinal dysbiosis either by microbiota transplantation, or introducing specific bacteria (animals procedure 1)

2. Behavioural tests

3. Treatment of gut dysbiosis either by diet or probiotics (animal procedure 3)

4. Behavioural tests

5. Measuring mitochondrial function or conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure *ex vivo* brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.
All experiments, except neuroimaging and measuring mitochondrial function, will be performed in isolators wherein the mice are housed to prevent colonisation with environmental bacteria. As we house mice in isolators we are restricted in options for behavioural tests. This excludes all equipment requiring power. In addition, we cannot use equipment to large to fit inside the isolators (larger than 45x45cm)
We will first introduce gut dysbiosis in mice. When we were succesfull in transmitting the ADHD-phenotype from humans to mice (animal procedure 1), we will introduce gut dysbiosis by microbiota transplantation. When we were unsuccesfull, we will introduce specific bacteria able to change behaviour and cognition to conventionally raised mice (animal procedure 1).
After introducing dysbiosis, we will conduct behavioural tests to investigate effects of dysbiosis on behaviour. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried

marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory. When we see changes in behaviour, we will treat these mice with diet. The exact nature of the diet used will depend on the results of animal procedure 3. After dietary intervention, we will conduct behavioural tests to see if the diet is able to ameliorate changes in behaviour.
We will focus on brain structure and function first. When we see changes in behaviour, brain structure, and brain function as result of one or more treatments, we will study mitochondrial function as well. Isoflurane, used to anaesthetise mice during neuroimaging to study brain function, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.
Mice used for neuroimaging techniques are sacrificed directly after scanning and their brains will be used to examine brain structure using histological and biochemical assays.

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

This experiment will take maximally 4 months. The aim of this experiment is to design dietary interventions able to normalise gut microbial composition and neurodevelopment. To study possible interventions, we first have to induce dysbiosis. We will do this by colonisation of mice with human microbiota or with specific bacteria. Any dysbiosis present in the donor microbiota will be transferred to the recipient mouse. We plan to use microbiota transplantation or by introduction of bacteria as method to change microbial composition, because introduced gut dysbiosis will be almost instant, long-term and with least side-effects. Inducing dysbiosis with diet will take three months and using antibiotics to promote gut dysbiosis will also introduce side effects. After colonisation we will conduct behavioural tests to study alterations in behaviour caused by dysbiosis. We then will give mice diets selected in animal procedure 3. We will again conduct behavioural tests to see if these diets are able to modify behaviour. Finally we will analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders.
This approach allows us to study dietary interventions to ameliorate traits of neurodevelopmental disorders. First, neurodevelopmental disorders are mimiced by inducing dysbiosis leading to altered behaviour and cognition. We then try to ameliorate these symptoms by giving mice high potential diets (selected in the third animal procedure).

In order to design interventions to ameliorate effects of the gut dysbiosis on neurodevelopment and function we first aim to induce gut dysbiosis in conventionally raised mice. First, we will induce intestinal dysbiosis either by transplantation of microbiota from individuals with ADHD transplantation to mice, or by introducing specific bacteria. When we were succesfull in transferring the ADHD-phenotype from individuals to mice, we will transplant germ-free mice with human microbiota. When we were unsuccesfull, we will introduce specific bacteria (see animal procedure 2) which were able to affect behaviour and cognition. We will colonise mice with a suspension of microbiota or bacteria in Phosphate Buffered Saline (PBS) via gavage. By oral administration, we can decrease variation as every mouse will receive exactly the same amount of

microbiota. Administration of bacterial suspensions via gavage will have a mild, short-term impact on the animals. This procedure will be done once and will take about 5 seconds per mouse.

After inducing dysbiosis, we will conduct behavioural tests (before and after inducing dysbiosis) which will take about 10 minutes each time. Behavioural tests can possibly cause mild, short-term distress. Tests conducted include the Open Field test, marble burying test, and object recognition test. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory.
When we see behavioural changes, we will treat mice with dietary interventions to ameliorate those symptoms using diets. The exact nature of the diet is dependent on outcomes of previous experiments (animal procedure 3). For example, we will give diets containing high saturated fat or unsaturated fatty acids, high caloric/sucrose, or diets containing probiotics. Mice will be fed diets ad libitum for approximately 3 months. During dietary intervention, we will conduct behavioural tests once a week to assess behavioural changes. We will use the same behavioural tests described above.
Three months after starting on the diet, we will investigate brain function and structure in one group of mice. For this, we will examine cerebral blood flow, connectivity structures, or grey and white matter integrity. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be sacrificed directly after these MRI experiments by transcardial perfusion-fixation using Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.
Only when we have seen altered mitochondrial function as result of dysbiosis (animal procedure 1-3) we will examine mitochondrial function in this experiment. In order to study whether dietary interventions are able to normalise brain mitochondrial function, we will measure mitochondrial function in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed in comparable conditions. Introducing microbiota via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests ($P<0.05$; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. All experiments are performed by experienced researchers or technicians. This experiment is only carried out when we were successful in transferring the ADHD-phenotype from humans to mice (animal procedure 1), or when were able to alter behaviour and cognition by introducing specific bacteria (animal procedure 2). Mitochondrial function will be measured only

when mitochondrial fucntion is proven to be affected by dysbiosis in previous experiments (animal procedure 1-3). Measuring mitochondrial function will only be performed in mice given the diet with the highest potential in affecting behaviour and cognition.

B. The animals

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

We will use germ-free mice (Mus musculus). Germ-free mice, mice without microbiota, are required for colonisation. These mice have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice, after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala. Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females.
G*Power is used to determine the appropriate sample size for these tests ($P<0.05$; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. The number of groups of mice used for this experiment depends on how many diets show potential in animal procedure 4. We will start with the diets showing highest potential in affecting behaviour and cognition. We will use test groups (N=14), as well as control groups (N=14). The maximum number of animals we consider to be necessary is 112 mice, allowing us to conduct 4 experiments to analyse altered behaviour, brain function, and brain structure. When mitochondrial function is altered in previous experiments, we will also use a group of mice to assess mitochondrial function (N=10). Measuring mitochondrial function will only be performed in mice given the diet with the highest potential in affecting behaviour and cognition. An additional 20 mice is required when mitochondrial function is proven to be affected by dysbiosis. The total number of animals we consider necessary is 132 mice for this experiment.

Species	Origin	Maximum number of animals	Life stage
Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	132	3 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:
  We plan to investigate effects of gut dysbiosis on behaviour, cognition and mitochondrial function. The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.
- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. This experiment is only carried out when previous experiments (animal procedures 1, 2, 4) were successful. We will only assess diets that were proven to be able to affect behaviour and cognition in animal procedure 4. Diets with the highest potentials are investigated first. We will only measure mitochondrial function in these mice when we demonstrated altered mitochondrial function in previous experiments. For mitochondrial function, we will only assess the diet most affecting behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Before we are able to ameliorate symptoms of neurodevelopmental disorders, we first have to induce them. In animal procedure 1 and 2 we will investigate the most appropriate method for inducing gut dysbiosis. We will either colonise mice with microbiota or introduce specific bacteria. Using one of these methods, introduced gut dysbiosis will be almost instant, long-term and with least side-effects. Inducing dysbiosis with diet will take three months and using antibiotics to promote gut dysbiosis will also introduce side effects. After inducing intestinal dysbiosis we will conduct behavioural tests to assess behavioural changes due to the dysbiosis. The used behavioural tests give as little as possible distress and pain, while still yielding reliable results. When we see behavioural changes in the animals we will try to ameliorate these changes by treating the

mice with dietary interventions. These interventions are chosen carefully in animal procedure 4. A group of control mice is given normal chow. Changes in behavioural are measured by the same behavioural tests used before the treatment. Finally cognition is assesed in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

[X] No

F. Accommodation and care

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

G. Location where the animals procedures are performed

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

[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, analgesiaor 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?

I. Other aspects compromising the welfare of the animals

Negative effects from colonisation with bacteria, mostly gastrointestinal problems like diarrhoea, may be expected. Behavioural and cognitive changes caused by the microbiota such as hyperactivity and increased anxiety. distress caused by these behavioural changes are considered to be mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Effects of the microbiota or bacteria on behaviour and cognition are described in literature. Some of the bacteria are known to occasionally cause diarrhoea in humans. Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them. Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

Transplanting human microbiota to germ-free mice has been done before (Ridaura, Faith et al. 2013; Bruce-Keller, Salbaum et al. 2015) where the researchers studied the role of gut microbiota on obesity. No adverse effects, except a significant increase in weight, were reported. Our goal is to study effects of microbiota from individuals with ADHD on cognition and behaviour in mice. Distress caused by the expected behavioural and cognitive changes is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 1%.

K. Classification of severity of procedures

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

The discomfort caused by the colonisation can be classified as mild to moderate. Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category 'non-recovery'.
Cumulative levels of discomfort are expected to be mild (80%) to moderate (20%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed in order to study brain function and structure, or brain mitochondrial function and structure.

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

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

[X] Yes

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer 2015-0077
2. Titel van het project: Elucidating the link between gut dysbiosis and mitochondrial dysfunction leading to neurodevelopmental disorders.
3. Titel van de NTS: Effecten van darmbacteriën op ontwikkelingsstoornissen zoals ADHD en autisme spectrum stoornis.
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: 23-04-2015
   - ☐ in vergadering besproken: 12-05-2015
   - ☐ vragen gesteld: 18-05-2015
   - ☐ antwoorden en aangepaste aanvraag ontvangen op 22-05-2015
   - ☐ in vergadering herbesproken: 02-06-2015
   - ☐ aanvraag compleet 02-06-2015
   - ☐ anderszins behandeld: aangepaste aanvraag en finaal advies zijn op 29 juli 2015 in een schriftelijke e-mailronde voorgelegd aan de DEC-leden voor instemming.
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: n.v.t.
   - ☐ advies aan CCD: 07-08-2015
7. Eventueel horen van aanvrager: n.v.t.
   - 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: 18-05-2015
   - Strekking van de vragen:
   - **Project Proposal:**
   - - 3.1 De onderzoekers postuleren dat verstoring van de darmflora op jonge leeftijd bijdraagt aan het ontstaan van ontwikkelingsstoornissen via dysfunctionerende mitochondria. De relatie tussen dysfunctionerende mitochondria en het ontstaan van ontwikkelingsstoornissen is aannemelijk gemaakt. De commissie vindt de aanwijzingen voor het effect van

darmbacteriën op mitochondria niet erg overtuigend. Is er meer evidentie te vinden voor deze relatie?

- - 3.4 Kiemvrije muizen hebben geen darmflora. Is er een effect op de hersenontwikkeling bij deze dieren, die leidt tot gedragsstoornissen? Zo nee, hoe past dit dan in uw hypothese? Is er evidentie dat de darmflora verschilt tussen mensen met ADHD en mensen zonder ADHD, en voor welke bacteriën verschilt dit? Waarom gebruiken de onderzoekers geen normale muizen waarop zij selectieve darmcontaminatie kunnen toepassen? In onderdeel 4 willen de onderzoekers de geïnduceerde ontwikkelingsstoornis behandelen met een dieet. Zoals beschreven duurt het langer voordat een dieet effect heeft op de samenstelling van de darmflora, waardoor de ontwikkeling van de muizenhersenen al is afgerond. Verwachten de onderzoekers dat zij met een dieet de ontwikkelingsstoornis op termijn kunnen genezen?

- **Description of Animal Procedures:**
- - DAP 1, onderdeel A eerste vraag: De onderbouwing voor het gebruik van de marble burying en de object recognition test ontbreekt. De onderzoekers worden verzocht dit in alle dierproeven (1-4) aan te passen.
- - DAP 1, onderdeel A tweede vraag: Wat is de maximale lengte van het experiment voor de dieren? Hoe gaan de onderzoekers de functie en structuur van mitochondria bestuderen en welke weefsels willen zij daarvoor gebruiken? De onderzoekers worden verzocht dit in alle dierproeven aan te passen.
- - DAP 1-3, onderdeel B: De onderzoekers gebruiken muizen van 3 weken oud, waarin een belangrijk deel van de hersenontwikkeling al heeft plaatsgevonden. Waarom starten zij het experiment niet vanaf de geboorte van de muizen? Kunnen de onderzoekers de aantallen dieren beter onderbouwen met behulp van het beoogde experimentele design? Kunnen zij beter uitleggen waarom zij respectievelijk 8, 5 en 4 experimenten willen doen?
- - DAP 2 en 3, onderdeel K: 20% van de muizen zal matig ongerief ervaren. Dit blijkt niet uit de gegeven beschrijving van het ongerief dat door de verschillende handelingen wordt veroorzaakt. Indien alle dieren licht ongerief zullen ervaren, dan worden de onderzoekers verzocht de percentages aan te passen, en indien nodig ook in de niet-technische samenvatting.
- - DAP 3, onderdeel A tweede vraag: Uit de gegeven beschrijving blijkt niet duidelijk hoe vaak de dieren de gedragstesten ondergaan: alleen bij de start en na drie maanden, of vaker? Er is sprake van gedragstesten voor en na kolonisatie, maar in dit experiment vindt geen kolonisatie plaats. Kunnen de onderzoekers toelichten waarom zij het dieet zo lang geven, wanneer er al na 3 weken effecten te meten zijn zoals in proef 1. Indien het zo lang duurt voordat het dieet effect heeft, verwachten de onderzoekers dan nog een effect op hersenontwikkeling? Wanneer zijn de hersenen van muizen volgroeid?
- - DAP4, onderdeel A eerste vraag: Verwachten de onderzoekers dat een ontwikkelingsstoornis een reversibele afwijking is die genezen kan worden door te interveniëren met een dieet? De onderzoekers worden gevraagd dit duidelijker uit te leggen.
- - DAP4, onderdeel A tweede vraag: De experimentele handelingen zijn hier preciezer omschreven. De onderzoekers worden verzocht dit ook te doen voor de andere dierproeven.

- Datum antwoord: 22-05-2015
- Strekking van de antwoorden:

- **Project proposal**
- 3.1 In de achtergrond van het projectvoorstel beschrijven wij de context van ons voorstel. Hier schrijven wij dat verstoring van darmmicrobiota op jonge leeftijd effecten heeft op ontwikkeling van het brein. Onze hypothese hierbij is dat de darmmicrobiota communiceert met het brein, onder andere via mitochondriën. Darmbacteriën produceren korte-keten vetzuren (Short-chain fatty acids; SCFA's) voornamelijk door fermentatie van koolhydraten. Wanneer de samenstelling van de darmmicrobiota verandert kan ook de samenstelling van microbiële metabolieten veranderen. De SCFA's worden opgenomen door darmepitheelcellen en kunnen door de bloedstroom vervoerd worden naar andere organen en weefsels. De commissie vindt deze aanwijzing voor het effect van darmmicrobiota op mitochondriën niet overtuigend. Wij zijn het er met de commissie eens dat dit punt niet erg duidelijk is en daarom hebben wij dit punt toegelicht in het projectvoorstel. Wij beschrijven hier drie aanwijzingen voor effecten van darmbacteriën op mitochondriën:

  1. Azijnzuur, propionzuur en boterzuur zijn de voornaamste korte-keten vetzuren geproduceerd door darmbacteriën. Deze SCFA's kunnen omgezet worden in de citroenzuurcyclus tot energierijke metabolieten (ATP, NADH en FAD) in mitochondriën. Boterzuur wordt voornamelijk gebruikt als energiebron voor cellen in de dikke darm. Azijnzuur en propionzuur worden getransporteerd naar andere delen van het lichaam. Azijnzuur wordt gebruikt door mitochondriën voor de synthese van lange-keten vetzuren (Christ, E.J. (1968) Fatty acid synthesis in mitochondria. Elongation of short-chain fatty acids and formation of unsaturated long-chain fatty acids. *Biochimica et biophysica acta,* **152,** 50-62, ibid.). Propionzuur wordt voornamelijk gebruikt als substraat voor gluconeogenese, een proces dat in de mitochondriën begint. Toediening van propionzuur aan ratten leidt tot cognitieve gebreken, verminderde sociale interacties, repetitief gedrag en abnormale motorische bewegingen (Shultz, S.R., MacFabe, D.F., Ossenkopp, K.P., Scratch, S., Whelan, J., Taylor, R. & Cain, D.P. (2008) Intracerebroventricular injection of propionic acid, an enteric bacterial metabolic end-product, impairs social behavior in the rat: implications for an animal model of autism. *Neuropharmacology,* **54,** 901-911). De vetzuren die worden gemaakt door darmbacteriën vervullen elk verschillende functies. Een veranderde samenstelling van bacteriën in de darmen kan leiden tot een veranderde samenstelling van geproduceerde SCFA's.
  2. SCFA's kunnen de werking van mitochondriën ook aantasten. Bijvoorbeeld apoptose te induceren in kankercellen van de dikke darm (Heerdt, B.G., Houston, M.A. & Augenlicht, L.H. (1997) Short-chain fatty acid-initiated cell cycle arrest and apoptosis of colonic epithelial cells is linked to mitochondrial function. *Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research,* **8,** 523-532.).
  3. Bovendien kunnen bacteriesoorten, zoals verschillende soorten *Pseudomonas*, de mitochondriële functie rechtstreeks aantasten door de mitochondriële surveillance pathway uit te schakelen. Mitochondriën, een rijke bron van cellulair ijzer, zijn aantrekkelijke doelwitten voor bacteriën aangezien vrij ijzer nauwelijks voorkomt in de bloedbaan (Huang, M.L., Lane, D.J. & Richardson, D.R. (2011) Mitochondrial mayhem: the mitochondrion as a modulator of iron metabolism and its role in disease. *Antioxidants & redox signaling,* **15,** 3003-3019.) IJzer is noodzakelijk voor processen zoals DNA-replicatie en metabolisme. Door het blokkeren van de surveillance pathway zijn andere virulentiefactoren zoals sideroforen, ijzer-bindende moleculen, effectiever in het aanvallen en daarmee verkrijgen van ijzer uit mitochondriën (Liu, Y., Samuel, B.S., Breen, P.C. & Ruvkun, G. (2014) Caenorhabditis elegans pathways that surveil and defend mitochondria. *Nature,* **508,** 406-410.). *Pseudomonas spp.*

zijn psychrotrophen, bacteriën die vermenigvuldigen bij lage temperaturen (0-4°C.). Deze bacteriën worden vaak aangetroffen in melk en melkproducten. Wij veronderstellen dat mensen deze bacteriën meer binnen krijgen dan tientallen jaren geleden doordat mensen deze producten vaker en langer bewaren in de koelkast dan enkele tientallen jaren geleden. Daarom willen wij de effecten van deze psychrothrophe bacteriën, zoals *Pseudomonas spp., Lactobacilus* en *Bifidobacterium,* op mitochondriën en breinontwikkeling onderzoeken.

- 3.4 Kiemvrije muizen vertonen abnormale breinfunctie en gedrag. Deze muizen vertonen bijvoorbeeld een abnormale reactie op stress. Deze stressreactie kan gecorrigeerd worden door de muizen te koloniseren met normale darmmicrobiota (Diaz Heijtz, R., Wang, S., Anuar, F., Qian, Y., Bjorkholm, B., Samuelsson, A., Hibberd, M.L., Forssberg, H. & Pettersson, S. (2011) Normal gut microbiota modulates brain development and behavior. *Proceedings of the National Academy of Sciences of the United States of America,* **108,** 3047-3052, en Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.N., Kubo, C. & Koga, Y. (2004) Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *The Journal of physiology,* **558,** 263-275). Ook hebben de darmbacteriën invloed op de ontwikkeling van het brein. Desbonnet *et al.* hebben volwassen muizen (55 tot 80 dagen oud) behandeld met een combinatie van verschillende antibiotica. De bacteriële diversiteit in de darmen werd hierdoor significant verminderd. In deze muizen was de cognitieve functie aangetast en konden de muizen minder goed onderscheid maken tussen bekende en onbekende voorwerpen. In het brein werd minder brain-derived neurotrophic factor (BDNF) aangemaakt, een zenuwcelstimulerende factor. BDNF speelt een belangrijke rol bij de vorming van nieuwe synapsen en is van belang voor cognitie (Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R.D., Cotter, P.D., Dinan, T.G. & Cryan, J.F. (2015) Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, behavior, and immunity*.). Kiemvrije muizen vertonen een vergelijkbaar fenotype. Deze tekst hebben wij toegevoegd aan het voorstel.

- Er is nog weinig onderzoek gedaan naar de darmmicrobiota van mensen met ADHD. Echter, uit persoonlijke communicatie met onze samenwerkende partners in dit onderzoek(naschrift ambtelijk secretaris: namen bekend bij de DEC) weten wij dat er verschillen zijn in samenstelling van darmmicrobiota tussen mensen met en mensen zonder ADHD. Deze bevindingen worden nu opgeschreven en daarna pas vrijgegeven. Daarnaast zijn er veel publicaties over de veranderde samenstelling van darmbacteriën in mensen met autisme. Onlangs is er een artikel gepubliceerd van Pärtty *et al.* waarin de auteurs beschrijven dat de darmmicrobiota van kinderen met ADHD anders is dan de darmmicrobiota van kinderen zonder ADHD. Zij beschrijven een vermindering van *Bifidobacterium* species in kinderen met ADHD (Partty, A., Kalliomaki, M., Wacklin, P., Salminen, S. & Isolauri, E. (2015) A possible link between early probiotic intervention and the risk of neuropsychiatric disorders later in childhood: a randomized trial. *Pediatric research*.). Tot nu toe is dit de enige studie gedaan naar de darmmicrobiota van mensen met ADHD.

- De commissie vraagt zich af waarom wij darmmicrobiota willen toedienen aan kiemvrije muizen in plaats van aan muizen met normale darmmicrobiota. Wij willen allereerst onderzoeken of wij gedrag en cognitie kunnen veranderen door kolonisatie met bepaalde darmmicrobiota. Gebruik van muizen met normale darmmicrobiota heeft als voordeel dat

deze muizen geen abnormale breinfunctie en gedrag vertonen zoals mogelijk de kiemvrije muizen. Het nadeel is dat deze muizen van zichzelf al een darmmicrobiota bezitten. Deze bestaande darmbacteriën zullen dan gaan concurreren met de toegediende microbiota en na verloop van tijd zal er een evenwicht ontstaan tussen bestaande en toegediende bacteriën. Hierdoor is het de vraag of wij effecten zullen zien van de toegediende darmbacteriën. Gebruik van kiemvrije muizen heeft als voordeel dat muizen 'schoon zijn' en binnen een korte tijd (1-3 dagen) gekoloniseerd zijn met de toegediende microbiota. Veranderingen in gedrag zijn gevolg van deze kolonisatie. Wij hebben deze motivatie voor het gebruik van kiemvrije dieren toegevoegd aan het voorstel. Wij willen ook het effect van bepaalde bacteriesoorten op gedrag en cognitie onderzoeken. Hiervoor willen we in eerste instantie ook kiemvrije muizen gebruiken om te onderzoeken wat het effect is van deze specifieke bacteriesoorten. Wanneer wij resultaat zien willen wij muizen met normale darmmicrobiota infecteren met deze bacteriesoorten om de natuurlijke situatie beter na te bootsen. Dit hebben wij ook aangepast in de aanvraag.

- In het projectvoorstel zijn wij van plan om ontwikkelingsstoornissen te induceren in muizen. Deze geïnduceerde ontwikkelingsstoornis willen wij vervolgens behandelen door middel van een dieet. De dierexperimentencommissie vraagt zich af of de ontwikkeling van de hersenen niet al is afgerond op het moment dat wij beginnen met de behandeling. Voeding heeft grote invloed op de darmmicrobiota en daarmee ook op de ontwikkeling van het brein. (Bos, D.J., Oranje, B., Veerhoek, E.S., Van Diepen, R.M., Weusten, J.M., Demmelmair, H., Koletzko, B., de Sain-van der Velden, M.G., Eilander, A., Hoeksma, M. & Durston, S. (2015) Reduced Symptoms of Inattention after Dietary Omega-3 Fatty Acid Supplementation in Boys with and without Attention Deficit/Hyperactivity Disorder. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*; Janssen, C.I., Zerbi, V., Mutsaers, M.P., de Jong, B.S., Wiesmann, M., Arnoldussen, I.A., Geenen, B., Heerschap, A., Muskiet, F.A., Jouni, Z.E., van Tol, E.A., Gross, G., Homberg, J.R., Berg, B.M. & Kiliaan, A.J. (2015) Impact of dietary n-3 polyunsaturated fatty acids on cognition, motor skills and hippocampal neurogenesis in developing C57BL/6J mice. *The Journal of nutritional biochemistry,* **26,** 24-35; Voreades, N., Kozil, A. & Weir, T.L. (2014) Diet and the development of the human intestinal microbiome. *Frontiers in microbiology,* **5,** 494.). Desbonnet *et al.* hebben volwassen muizen (55 tot 80 dagen oud) behandeld met een combinatie van verschillende antibiotica. De auteurs beschrijven een aangetaste cognitieve functie in deze muizen (Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R.D., Cotter, P.D., Dinan, T.G. & Cryan, J.F. (2015) Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, behavior, and immunity).* Het brein is een plastisch orgaan, hersencellen blijven zich aanpassen aan externe en interne stimuli. Wij verwachten dat wij door middel van dieet bepaalde symptomen van ontwikkelingsstoornissen, zoals angst en abnormale bewegingsactiviteit, te kunnen verminderen.

- **Description of Animal Procedures**

- DAP1, onderdeel A eerste vraag: Hartelijk dank voor deze opmerkzaamheid. Wij hebben de onderbouwing voor de marble burying test en de object recognition test toegevoegd aan de dierexperimenten.

- DAP 1, onderdeel A tweede vraag: De commissie vraagt zich af wat de maximale duur van dit experiment zal zijn. In deze procedure worden de kiemvrije dieren gekoloniseerd met darmmicrobiota ofwel specifieke bacteriën. Na kolonisatie worden gedragstesten afgenomen en aan het einde van het experiment wordt cognitie onderzocht door middel van MRI. De maximale lengte van dit experiment zal vier weken zijn.

- De muizen zullen worden geofferd via cervicale dislocatie waarna mitochondriële functie en structuur onderzocht zullen worden in het hersenweefsel. Mitochondriële functie zal worden onderzocht door protocollen die hier op de afdeling veel gebruikt worden. Deze protocollen omvatten testen voor respiratie, complex I-IV activiteit, mitochondrieel membraan potentiaal en ATP/ADP levels. Mitochondriële structuur wordt door histochemische technieken onderzocht in gefixeerd hersenweefsel. Al deze testen worden post mortem uitgevoerd.

- DAP 1-3, onderdeel B: Wij vinden dat de commissie hier gelijk heeft qua breinontwikkeling en dat het gebruik van jongere muizen voorkeur geniet. Wij willen echter muizen van ongeveer 3 weken oud gebruiken voor dit experiment omdat gavage bij jongere dieren lastig is en tot hoger ongerief leidt dan bij muizen van 3 weken oud. Gavage is de voorkeursmethode voor het toedienen van darmmicrobiota om de variatie zo klein mogelijk te houden. Hierdoor zijn minder muizen nodig. Wij starten de experimenten in muizen van drie weken oud, dit is voor het begin van de adolescentie (begint omstreeks 6-7 weken). In deze periode is het brein nog heel plastisch: er vindt een overproductie van axonen en synapsen plaats en een toename in dichtheid van connecties tussen de amygdala and prefrontale cortex (Casey, B.J., Getz, S. & Galvan, A. (2008) The adolescent brain. *Developmental review : DR,* **28,** 62-77.) Uit andere onderzoeken blijkt dat antibioticagebruik of dieet ook op latere leeftijd nog effecten heeft op breinfunctie (Desbonnet, L., Clarke, G., Traplin, A., O'Sullivan, O., Crispie, F., Moloney, R.D., Cotter, P.D., Dinan, T.G. & Cryan, J.F. (2015) Gut microbiota depletion from early adolescence in mice: Implications for brain and behaviour. *Brain, behavior, and immunity*). Daarom verwachten wij ook effect te zien van darmmicrobiota in muizen van 3 weken oud.

- DAP1, onderdeel B:De commissie vraagt om betere onderbouwing van de aantallen dieren per groep. Aan de hand van eerder uitgevoerde experimenten binnen onze onderzoeksgroep is vastgesteld dat 14 muizen per groep nodig is om verschillen aan te kunnen tonen voor de te onderzoeken parameters. Dit aantal is gebaseerd op poweranalyses gebaseerd op gegevens gevonden in literatuur en uit onze voorgaande studies. De commissie vraagt tevens waarom wij 8 experimenten willen doen. In deze dierproef worden twee componenten van ons onderzoeksvoorstel onderzocht. De eerste vraag die wij willen beantwoorden is of de darmmicrobiota effecten heeft op gedrag en cognitie. Om dit te onderzoeken moeten we één experiment uitvoeren. De darmmicrobiota is afkomstig van mensen die gediagnosticeerd zijn met ADHD. Wij hebben hiervoor mensen geselecteerd die extreme hyperactiviteit vertonen om grotere gedragsverschillen te genereren en te detecteren in onze proefopzet. De tweede vraag die wij willen onderzoeken is of specifieke bacteriën een effect hebben op cognitie en gedrag. Voor het beantwoorden van deze vraag moeten we in totaal 7 experimenten uitvoeren. Eerst willen we vier verschillende bacteriestammen onderzoeken, zoals *Lactobacillus* en *Bifidobacterium,* die worden vaak gebruikt als probiotica, (Gilbert, J.A.,

Krajmalnik-Brown, R., Porazinska, D.L., Weiss, S.J. & Knight, R. (2013) Toward effective probiotics for autism and other neurodevelopmental disorders. *Cell,* **155,** 1446-1448) en *Pseudomonas spp,* die vaak voorkomen in melk en melkproducten. Kiemvrije muizen zullen hiervoor worden gekoloniseerd met deze bacteriesoorten waarna gedrag en cognitie zullen worden onderzocht. Vervolgens zullen wij drie experimenten uitvoeren waarin we muizen met normale darmmicrobiota infecteren met de drie bacteriestammen die het meeste effect hebben op gedrag en cognitie.

- DAP 2, onderdeel B: De commissie vraagt om betere onderbouwing van de aantallen dieren per groep. Aan de hand van eerder uitgevoerde experimenten binnen onze onderzoeksgroep is vastgesteld dat 14 muizen per groep nodig is om verschillen aan te kunnen tonen voor de te onderzoeken parameters. Dit aantal is gebaseerd op poweranalyses gebaseerd op gegevens gevonden in literatuur en uit onze voorgaande studies. De dierexperimenten-commissie vraagt ook om nadere uitleg over onze keuze om maximaal 5 experimenten te willen doen. In deze dierproefbeschrijving willen wij de effecten van antibiotica op darmmicrobiota en breinontwikkeling onderzoeken. Wij hebben vijf klassen antibiotica geselecteerd op basis van literatuur die wij graag zouden willen onderzoeken. Om deze klassen antibiotica te testen moeten wij vijf experimenten uitvoeren. Deze selecteerde klassen worden veel voorgeschreven aan jonge kinderen en daarom is het belangrijk om van deze vijf klassen de effecten te onderzoeken op darmmicrobiota en breinontwikkeling.

- DAP 2 en 3, onderdeel K: De dierexperimentencommissie stelt dat het cumulatieve ongerief niet blijkt uit de beschrijving van de verschillende handelingen. De onderzoekers hebben de beschrijving en de bijlage IV van richtlijn 2010/63/EU nogmaals nagelezen en hebben hieruit geconcludeerd dat het cumulatieve ongerief van alle dieren licht is. Wij verwachten dat de behandeling met antibiotica hooguit licht ongerief veroorzaakt. Geen van de klassen antibiotica zal matig ongerief veroorzaken. Dit hebben wij aangepast in onze aanvraag. Wij verwachten dat de diëten, die wij willen onderzoeken, licht ongerief veroorzaken aangezien het geen restrictieve diëten zijn. Dit hebben wij aangepast in onze aanvraag.

- DAP 3, onderdeel A tweede vraag : Wij zijn niet duidelijk geweest over hoe vaak de dieren gedragstesten ondergaan en hebben dit aangepast in de dierproefbeschrijvingen. De muizen ondergaan gedragstesten maximaal eenmaal per week. Verder vragen de commissieleden zich af waarom wij de dieren zo lang behandelen aangezien in het eerste experiment al na 3 weken effect te zien is. De muizen die wij van plan zijn te gebruiken in dit experiment hebben een normale darmmicrobiota. Het kan lang duren, volgens de literatuur zo'n 1-2,5 maand, om deze microbiota te veranderen door middel van dieet. In het eerste experiment willen wij kiemvrije dieren gebruiken, daarin kan de microbiota bijna onmiddellijk veranderd worden door toediening van darmmicrobiota. Uit andere onderzoeken blijkt dat dieet ook op latere leeftijd nog effecten heeft op breinfunctie. Dus verwachten wij ook effecten te zien op breinontwikkeling van dieet op latere leeftijd. Dit is ook een doel van het experiment, het onderzoeken of een dieetinterventie op latere leeftijd nog de breinfunctie kan beïnvloeden. Wij starten de experimenten in muizen van drie weken oud, dit is voor het begin van de adolescentie (begint omstreeks 6-7 weken). In deze periode is het brein nog heel plastisch: er vindt een overproductie van axonen en synapsen plaats en een toename in dichtheid van connecties tussen de amygdala and prefrontale cortex (Casey, B.J., Getz, S. & Galvan, A.

(2008) The adolescent brain. *Developmental review : DR,* **28,** 62-77). Deze snelle groei gaat door tot de muizen ongeveer 3 maanden oud zijn. Volgens de Jackson Laboratory, een leverancier van muizen, zijn muizen volwassen tussen 3-6 maanden. In deze periode is het brein volgroeid, maar is nog beïnvloedbaar door verschillende factoren.

- DAP 3, onderdeel B: De commissie vraagt om betere onderbouwing van de aantallen dieren per groep. Aan de hand van eerder uitgevoerde experimenten binnen onze onderzoeksgroep is vastgesteld dat 14 muizen per groep nodig is om verschillen aan te kunnen tonen voor de te onderzoeken parameters. Dit aantal is gebaseerd op poweranalyses gebaseerd op gegevens gevonden in literatuur en uit onze voorgaande studies. De commissie vraagt ook of wij beter kunnen uitleggen waarom wij maximaal 4 experimenten willen uitvoeren. In deze dierproef willen wij graag onderzoeken wat het effect is van verschillende diëten op darmmicrobiota en breinontwikkeling. Wij hebben vier diëten geselecteerd op basis van literatuur. Deze geselecteerde diëten omvatten diëten met hoge concentraties verzadigde of onverzadigde vetten en calorierijke diëten. Met een maximum van vier experimenten kunnen wij de effecten van deze diëten onderzoeken.

- DAP4, onderdeel A eerste vraag: De commissie vraagt of wij verwachten dat een ontwikkelingsstoornis een reversibele afwijking is die genezen kan worden door middel van dieet. Onze verwachtingen hadden wij niet duidelijk opgeschreven. Wij verwachten deze ontwikkelingsstoornissen niet te kunnen genezen door middel van dieet, maar wij verwachten wel symptomen van ontwikkelingsstoornissen op deze manier te kunnen verminderen.

- DAP4, onderdeel A tweede vraag: De dierexperimentencommissie vindt de experimentele handelingen in dit experiment preciezer beschreven. Wij hebben de andere experimenten ook aangepast.
- 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.

C. Beoordeling (inhoud):

1. Het project is:

□□ uit wetenschappelijk oogpunt verantwoord.

2. De in de aanvraag aangekruiste doelcategorie, translationeel onderzoek, is slechts gedeeltelijk in overeenstemming met de hoofddoelstelling. De DEC is van oordeel dat dit project voor een aanzienlijk deel betrekking heeft op fundamenteel onderzoek. Daarnaast wordt er verkennend onderzoek gedaan naar de mogelijkheden om de bevindingen te vertalen in concrete interventies. De DEC meent dat dit verschil van inzicht over de doelcategorie de ethische afweging die zij dient te maken niet beïnvloedt.

3. De DEC onderschrijft het belang van het voorgenomen onderzoek, dat er op gericht is om de betrokkenheid van de bacteriën in de darm bij het ontstaan en verloop van (neuronale) ontwikkelingsstoornissen, zoals ADHD en Autisme Spectrum Stoornis (ASD), in kaart te brengen. De doelstelling is nader uitgewerkt in de volgende subdoelstellingen: 'rol van de darmflora of specifieke bacteriën bij gedrag en cognitie'; 'effect van een onbalans in de darmflora op het functioneren van de mitochondriën; 'effect van diëten en antimicrobiële behandelingen op gedrag en cognitie'; 'ontwerpen van antimicrobiële behandelingen en diëten om de samenstelling van de darmflora te normaliseren en zo te komen tot een normaal functioneren van de mitochondriën en normale ontwikkeling van de hersenen'. De hypothese van dit onderzoek is dat de samenstelling van het microbioom in de darm het functioneren van de mitochondriën beïnvloedt en via die route bij zou kunnen dragen aan het ontstaan van ontwikkelingsstoornissen van de hersenen, zoals ADHD en ASD. Het onderzoek heeft in de ogen van de DEC weliswaar betrekking op een risicovolle hypothese, maar daarbij dient in aanmerking te worden genomen dat de voorgestelde dierproeven voor het overgrote deel van de dieren licht ongerief zullen veroorzaken. De te behalen onderzoeksresultaten zullen meer inzicht verschaffen in het veronderstelde effect van darmbacteriën op mitochondria en op cognitie en gedrag. Voorts zal het voorgestelde onderzoek meer inzicht verschaffen in het effect van dieet aanpassingen of antimicrobiële behandeling op darmflora, cognitie, gedrag, hersenstructuren en –functie en mitochondriële functie. Deze resultaten kunnen op termijn bijdragen aan de ontwikkeling van antimicrobiële- of dieetinterventies voor de doelgroep om via beter functionerende mitochondria de ernst van ontwikkelingsstoornissen te verminderen. Het aantrekkelijk hieraan is dat het om relatief eenvoudig te implementeren, weinig ingrijpende interventies gaat.

De grote omvang van de maatschappelijke problematiek verbonden met ontwikkelingsstoornissen als ADHD en ASD, brengt de DEC tot het oordeel dat zowel het verwerven van inzicht in de factoren die bijdragen aan het ontstaan ervan, als het uitzicht op eenvoudig te implementeren interventies, een substantieel belang vertegenwoordigen.

4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Het langs verschillende wegen beïnvloeden van de samenstelling van het microbioom in de darmen van muizen zal inzicht verschaffen in de invloed van het microbioom op het functioneren van de mitochondriën en op de hersenontwikkeling. Ook zal inzicht verkregen worden in de mogelijkheden om via dieetmaatregelen en behandeling met antibiotica het microbioom zo te beïnvloeden dat de functie van de mitochondriën en de hersenontwikkeling worden genormaliseerd. In de aanpak zijn verschillende, goed gekozen

go/no go momenten ingebouwd. Deze onderzoeksgroep heeft voldoende ervaring in dit onderzoeksveld en met de voorgestelde dierproeven. De gekozen aanpak leidt tot betrouwbare uitspraken over het effect van darmflora op cognitie, op gedrag (aandacht, hyperactiviteit en impulsiviteit) en op hersenstructuren en –functie van muizen.

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. Tien procent van de dieren in dit onderzoek zal matig ongerief ondervinden. De overige dieren ondervinden licht ongerief. Bij de dieren die matig ongerief ondervinden is dat hoofdzakelijk het gevolg van het toedienen van antibiotica of humane darmflora via orale gavage en de directe gevolgen daarvan (tijdelijke diarree) voor het welzijn. Bij de dieren die licht ongerief ondergaan is dat hoofdzakelijk het gevolg van de toediening van antibiotica, een aangepast dieet en het uitvoeren van gedragstesten.
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. Het effect van darmbacteriën op cognitie, gedrag en hersenstructuren en –functie is niet zonder proefdieren te onderzoeken. De onderzoekers kiezen voor de muis als modeldier omdat het gedragsrepertoire van dit dier voldoende uitgebreid is om verstoringen in gedrag te kunnen meten.
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. Metingen van mitochondriële functies waarvoor extra dieren nodig zijn, worden tot een minimum beperkt. De onderzoekers hebben go/no go momenten ingebouwd om onnodige dierproeven te voorkomen. De DEC is het eens met het beschreven onderzoeksmodel en de onderbouwing van het aantal benodigde dieren, en is van oordeel dat het project kan worden uitgevoerd met maximaal 750 muizen.
9. Het project is in overeenstemming met de vereiste van de **verfijning** van dierproeven. De experimentele handelingen bij de dieren zullen worden uitgevoerd door hierin getrainde onderzoekers, waardoor de stress voor de dieren zoveel mogelijk wordt beperkt. De voorgestelde gedragsexperimenten veroorzaken slechts licht ongerief bij de dieren. De dieren ontwaken niet meer uit hun verdoving. 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 kunnen belangrijke nieuwe inzichten worden verkregen in het effect van de samenstelling van de darmflora op het functioneren van de mitochondriën, de ontwikkeling van de hersenen en cognitie en gedrag. Ook wordt onderzocht of en hoe antimicrobiële behandelingen en dieetinterventies deze factoren beïnvloeden. Op termijn zou dit kunnen bijdragen aan het ontwikkelen van antimicrobiële of dieetinterventies die de ernst van ontwikkelingsstoornissen zoals ADHD en ASD kunnen verminderen. De interventies waaraan men denkt zouden relatief eenvoudig

geïmplementeerd kunnen worden. De grote omvang van de maatschappelijke problematiek verbonden met ontwikkelingsstoornissen als ADHD en ASD, brengt de DEC tot het oordeel dat zowel het verwerven van inzicht in de factoren die bijdragen aan het ontstaan ervan, als het uitzicht op eenvoudig te implementeren interventies, een substantieel belang vertegenwoordigen.

Tegenover dit substantiële belang staat het gegeven dat ongeveer 10% van de maximaal 750 muizen matig ongerief zal ondervinden, omdat, in een aantal gevallen via orale gavage, antibiotica en humane darmflora wordt toegediend. Dit kan tijdelijk tot ziekte en diarree leiden. De overige dieren ondervinden licht ongerief. De commissie is er van overtuigd dat bij de dierproeven adequaat invulling zal worden gegeven aan de vereisten op het gebied van de vervanging, vermindering en/of verfijning van dierproeven. Het resterende ongerief is onvermijdelijk, wil men de doelstellingen kunnen realiseren.

De DEC is van oordeel dat het hier boven geschetste belang de onvermijdelijke, voor het merendeel licht 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

2. Het uitgebrachte advies is gebaseerd op consensus.

Centrale Commissie Dierproeven

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Aanvraagnummer
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Bijlagen
2

Datum 11-08-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 8 augustus 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002015208. 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. Zodra uw aanvraag compleet is, ontvangt u binnen veertig werkdagen een beslissing op uw aanvraag. 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 wordt uw aanvraag buiten behandeling 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.zbo-ccd.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
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	41055629
Straat en huisnummer:	[redacted]
Postbus:	9102
Postcode en plaats:	6525 EZ NIJMEGEN
IBAN:	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer:	UMC St Radboud

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

Gegevens verantwoordelijke uitvoering proces

Naam:	[redacted]
Functie:	Instantie voor Dierenwelzijn
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Over uw aanvraag

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

Over uw project

Geplande startdatum:	8 september 2015
Geplande einddatum:	8 september 2019
Titel project:	Elucidating the link between environmental factors and mitochondral dysfunction leading
Titel niet-technische samenvatting:	effecten van de darmmicrobiota op ontwikkelingsstoornissen
Naam DEC:	RU DEC
Postadres DEC:	Postbus 9101, 6500 HB Nijmegen ([redacted]
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 741,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

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

Ondertekening

Naam:	[redacted]
Functie:	Instatie voor dierenwelzijn
Plaats:	Nijmegen
Datum:	8 augustus 2015

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Katholieke Universiteit Nijmegen
P [redacted]
Postbus 9102
6525 EZ NIJMEGEN

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

Onze referentie
Aanvraagnummer
AVD103002015208

Bijlagen
2

Datum 11-08-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 11 augustus 2015
Vervaldatum: 10 september 2015
Factuurnummer: 201570208

Omschrijving	**Bedrag**
Betaling leges projectvegrunning dierproeven Betreft aanvraag AVD103002015208	€ 741,00

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

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Katholieke Universiteit Nijmegen
t.a.v. [redacted]
Geert Grroteplein-Noord 10
6500HB Nijmegen

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

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

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD103002015208

Uw referentie

Bijlagen

Datum 17-08-2015
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 08 augustus hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Effecten van de darmmicrobiota op ontwikkelingsstoornissen' met aanvraagnummer AVD103002015208. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Niet technische samenvatting

1. De niet technische samenvatting bij uw aanvraag geeft een ander aantal dieren (750) weer dan de bijlages dierproeven (768). Geeft het correcte aantal dieren op en corrigeer het aantal waar nodig.

2. U noemt in de bijlage dierproef knockout dieren. Voor het fokken/houden en gebruiken van GGO dieren die niet uit een established line komen is een vergunning vereist. Ook voor het creëren van een lijn of houden van een lijn waarvan dieren ongerief hebben is een vergunning vereist. Daarom hebben wij hierover de volgende vragen:
   a. Bent u van plan GGO dieren te gaan gebruiken, zo kunt u toelichten welke en waarom?
   b. Gaat het daarbij om zogenaamde established lines of worden er lijnen gecreëerd?
   c. Indien het gaat om nieuw gecreëerde lijnen, worden deze lijnen in de instelling gecreëerd of naar de instelling toegehaald?
   d. Is er sprake van ongerief van deze dieren in de fok?

3. U gaat in experiment 1 oefenen op surplus muizen. Wat zijn de leerdoelen per training? Valt te verwachten dat voor de dieren van ander of meer ongerief sprake is, bijvoorbeeld het oefenen met oral gavages? Waarom

wordt dit geoefend? Gaat het om onderzoekers die de technieken nog niet beheersen? In dat geval moet ook de categorie hoger onderwijs en educatie worden toegevoegd in het projectvoorstel.

Wij vragen u deze informatie te verduidelijken.

Leges
De leges die u verschuldigd bent zijn nog niet door ons ontvangen of de betaling is nog niet verwerkt. Uw aanvraag is niet compleet als de leges niet zijn ontvangen.

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

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van deze brief op. U kunt dit aanleveren via NetFTP. Stuurt u het per post op, gebruik dan het formulier dat u bij deze brief krijgt. Als u de antwoorden op de vragen uiterlijk woensdag 19 augustus 2015 verstuurd, kan de CCD deze nog meenemen in haar vergadering van 28 augustus 2015.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlage:
- formulier Melding Bijlagen via de post

19 AUG 2015

Radboud universitair medisch centrum
Anatomie

Postbus 9101, 6500 HB Nijmegen

[redacted]

[redacted]

www.radboudumc.nl

KvK 41055629/4

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

Datum	Ons kenmerk	Pagina
18 augustus 2015		1 van 1

Onderwerp
Aanvulling Aanvraag projectvergunning dierproeven. Aanvraagnummer: AVD103002015208

Radboudumc

Radboud universitair medisch centrum
[redacted]

Postbus 9101, 6500 HB Nijmegen
[redacted]

T (024) 36 13341
F (024) 36 13789

[redacted]

www.radboudumc.nl

Datum	Ons kenmerk	Pagina	
18 augustus 2015		1 van 3	KvK 41055629/4

Onderwerp
Aanvulling Aanvraag projectvergunning dierproeven. Aanvraagnummer: AVD103002015208

Wij willen de Centrale Diercommissie van harte danken voor de tijd die zij gestoken hebben in het lezen en beoordelen van deze aanvraag. Wij hebben de opmerkingen en vragen van de commissie getracht zo goed mogelijk te beantwoorden en te verwerken in het projectvoorstel en in de beschrijving van de dierproeven (zie bijlagen) en hopen dat het voorstel hiermee goedgekeurd kan worden.

Projectvoorstel

Vraag:
De niet-technische samenvatting bij uw aanvraag geeft een ander aantal dieren (750) weer dan de bijlages dierproeven (768). Geeft het correcte aantal dieren op en corrigeer het aantal waar nodig.

Antwoord:
U merkt op dat het opgegeven aantal muizen in de niet-technische samenvatting niet overeen komt met het gegeven aantal muizen in het projectvoorstel. Hartelijk dank voor uw opmerkzaamheid. Wij hebben het aantal muizen in de niet-technische samenvatting aangepast.

Vraag:
U noemt in de bijlage dierproef knockout dieren. Voor het fokken/houden en gebruiken van GGO dieren die niet uit een established line komen is een vergunning vereist. Ook voor het creëren van een lijn of houden van een lijn waarvan dieren ongerief hebben is een vergunning vereist. Daarom hebben wij hierover de volgende vragen:

a. *Bent u van plan GGO dieren te gaan gebruiken, zo kunt u toelichten welke en waarom?*
b. *Gaat het daarbij om zogenaamde established lines of worden er lijnen gecreëerd?*
c. *Indien het gaat om nieuw gecreëerde lijnen, worden deze lijnen in de instelling gecreëerd of naar de instelling toegehaald?*
d. *Is er sprake van ongerief van deze dieren in de fok?*

Antwoord:

a) Wij zijn van plan om in samenwerking met TNO Leiden ook de LDLr-/- muizen te gebruiken en hebben hiervoor een GGO vergunning (IG 04-002). Op een chow dieet ontwikkelen deze muizen geen obesitas. Maar wanneer zij gevoed worden met specifieke energierijke diëten ontwikkelen deze muizen obesitas. Obesitas tijdens zwangerschap is geassocieerd met mitochondriële dysfuncties in eicellen. [1] En daarom willen wij deze muizen gaan gebruiken om te onderzoeken wat effecten zijn van obesitas in een vroege levensfase op darmmicrobiota, mitochondriën en breinontwikkeling en of wij deze effecten kunnen verminderen met nutritionele interventie of antibiotica, aangezien deze behandelingen de darmmicrobiota, mitochondriën en breinontwikkeling mogelijk kunnen beïnvloeden.
b) De LDLr-/- muis is een established line ontwikkeld als model voor metabole ziekten door TNO Leiden.
c) Het gaat hier niet om een nieuw gecreëerde lijn en de dieren zullen vanuit TNO Leiden voor het experiment getransporteerd worden naar het Centrale DierenLaboratorium (CDL) in Nijmegen. Zoals vermeld hebben wij een GGO vergunning hiervoor (IG 04-002).
d) De LDLr-/- muis heeft geen extra ongerief, niet in de fok en niet bij supplementatie van (energierijke) diëten. Dit blijkt uit een eerder goedgekeurde dierexperimentele aanvraag (zie bijgevoegde Aanvraag onder 4: Aard van het ongerief; en ook Radonjic *et al.* (2013)[2]).

Vraag:
U gaat in experiment 1 oefenen op surplus muizen. Wat zijn de leerdoelen per training? Valt te verwachten dat voor de dieren van ander of meer ongerief sprake is, bijvoorbeeld het oefenen met oral gavages? Waarom wordt dit geoefend? Gaat het om onderzoekers die de technieken nog niet beheersen? In dat geval moet ook de categorie hoger onderwijs en educatie worden toegevoegd in het projectvoorstel.

Antwoord:
Het valt niet te verwachten dat het oefenen van technieken op de muizen voor meer ongerief zorgt. De onderzoekers hebben voldoende ervaring met de uit te voeren technieken. Maar tijdens deze experimenten gaan wij jonge dieren gebruiken. Hiervoor moet een aantal testen in de MRI set-up gedaan worden om te zien of optimale scans verworven kunnen worden of dat positie van bijvoorbeeld coil aangepast moet worden. Verder wordt voor MRI-testen algehele anesthesie toegepast waaruit de dieren niet zullen bijkomen. Hiervoor zullen wij isofluraan gebruiken. De juiste concentratie isofluraan moet worden aangepast aan het gewicht van de muizen. De dosis anesthesie is van invloed op de hersendoorbloeding en heeft mogelijk ook effect op mitochondriële functie. Door een aantal surplus muizen te gebruiken om de juiste set-up en isofluraanconcentraties vast te stellen, kunnen wij de optimale standaardcondities bepalen voor de neuroimaging. De onderzoekers hebben ervaring met het toedienen van microbiota via gavage. Maar de muizen die gavage ondergaan in deze experimenten worden gehuisvest in isolatoren. Met behulp

---

[1] Wu, L.L., Russell, D.L., Wong, S.L., Chen, M., Tsai, T.S., St John, J.C., Norman, R.J., Febbraio, M.A., Carroll, J. & Robker, R.L. (2015) Mitochondrial dysfunction in oocytes of obese mothers: transmission to offspring and reversal by pharmacological endoplasmic reticulum stress inhibitors. *Development,* **142,** 681-691.

[2] Radonjic, M., Wielinga, P.Y., Wopereis, S., Kelder, T., Goelela, V.S., Verschuren, L., Toet, K., van Duyvenvoorde, W., van der Werff van der Vat, B., Stroeve, J.H., Cnubben, N., Kooistra, T., van Ommen, B. & Kleemann, R. (2013) Differential effects of drug interventions and dietary lifestyle in developing type 2 diabetes and complications: a systems biology analysis in LDLr-/- mice. *PloS one,* **8,** e56122.

van surplus muizen kunnen de onderzoekers hun vaardigheden, wat betreft gavage en gedragsobservaties optimaliseren voor uitvoering in isolatoren.

Leges

Vraag:
De leges die u verschuldigd bent zijn nog niet door ons ontvangen of de betaling is nog niet verwerkt. Uw aanvraag is niet compleet als de leges niet zijn ontvangen. Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen omdat de gegevens onvolledig of onduidelijk zijn.

Antwoord:
Wij hebben begrepen dat de leges die wij u verschuldigd zijn nog niet zijn ontvangen. Wij hebben hierover contact opgenomen met ons lokale DEC (RU-DEC) en hebben begrepen dat er een miscommunicatie was omtrent de factuur. Inmiddels hebben wij de gecorrigeerde factuur ontvangen en hebben wij de betaling in gang gezet. Wij hebben hierover contact gehad m[redacted]en hij heeft toestemming gegeven voor uitstel van betaling met een paar dagen.

Wij hopen dat deze beantwoording van vragen en de gecorrigeerde aanvraag opnieuw in behandeling genomen kan worden.

Met vriendelijke groet,

[redacted]

19 AUG 2015

Centrale Commissie Dierproeven

Melding

Bijlagen via de post

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

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager [redacted]

Postcode 6500 HB [redacted]

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

Aanvraagnummer AVD103002015208

2 Bijlagen

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

☒ Aanvullende informatie

☒ Aanvraag eerder goedgekeurd voorstel

☒ Project proposal en description animal procedures

3 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam [redacted]

Datum 10 - 08 - 2015

Handtekening [redacted]

1□.

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.zbo-ccd.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.	Elucidating the link between gut dysbiosis and mitochondrial dysfunction leading to neurodevelopmental disorders

2 Categories

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

[] Basic Research
[X] Translational or applied research
[] Regulatory use of 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.

Neurodevelopmental disorders are disabilities associated mainly with the functioning of the neurological system and brain. Individuals with these disorders can experience difficulties with language and speech, behaviour, learning, motor skills, and other neurological functions. Most neurodevelopmental disorders are caused by genetic abnormalities, including fragile-X syndrome and Down syndrome. Other neurodevelopmental disorders are referred to as complex because they have multiple and complex contributors rather than one clear cause. These complex disorders typically involve cognitive, behavioural or personality characteristics (Tager-Flusberg, 1999a). Complex neurodevelopmental disorders, such as attention-deficit/hyperactivity disorder (ADHD) and autism spectrum disorder (ASD), are common and affect both children and adults.
Development of the nervous system is a complex process involving differentiation of neurons from neural stem cells. These differentiating neurons require high levels of energy, generated by mitochondria in the form of adenosine triphosphate (ATP). Mitochondria are localised in synapses, and synaptic function can be disturbed by mitochondrial morphology, function, and alterations in amount of mitochondria per cell (Kageyama and Wong-Riley 1982). Mitochondrial dysfunction contributes to several neurodevelopmental diseases (Anitha, Nakamura et al. 2013). Prevalence of several co-morbid features, like learning disabilities, motor delay, developmental regression, seizures and gastrointestinal (GI) dysfunctions, is typically higher in people with both a neurodevelopmental disorder and mitochondrial dysfunction (Rossignol and Frye 2012; Hsiao, McBride et al. 2013). Induced mitochondrial dysfunction in rats led to certain behavioural, metabolic and brain changes consistent with several neurodevelopmental disorders. These changes include repetitive behaviours, hyperactivity, increased amounts of reactive oxidative stress (ROS), reduced levels of antioxidants, and microglial activation (Rodríguez-capote et al. 2008).
GI dysfunction, such as chronic diarrhoea, constipation or intestinal infection, is a co-morbidity of special interest given its high prevalence and high correlation with symptom severity in several neurodevelopmental disorders (Adams, Johansen et al. 2011). The mechanisms leading to these GI problems remain unclear. One of the explanations of these GI dysfunctions found in people with neurodevelopmental disorders is dysbiosis, a significant change in gut bacterial composition. Gut bacteria contribute to neurodevelopment and function (Cryan and Dinan 2012) and there is a growing number of studies reporting dysbiosis in individuals diagnosed with neurodevelopmental disorders (Finegold, Downes et al. 2012; Gondalia, Palombo et al. 2012; Williams, Hornig et al. 2012; Kang, Park et al. 2013; Borre, O'Keeffe et al. 2014).

The adult human (and mouse) gut microbiota is dominated by the bacterial phyla Firmicutes and Bacteriodetes and seems to be stable and resilient against short-term changes (Faith, Guruge et al. 2013). The infant gut microbiota on the other hand is less stable and stabilises when the infant is 2-3 years old. The infant gut microbiota can be influenced by multiple factors, including antibiotics administered to the infant or mother, level of breastfeeding, mode of delivery, and genetics (Fallani, Amarri et al. 2011). Bergström et al. studied the gut microbiota of infants in a three-year Danish study with a cohort of 330 infants. Infants between 9 and 18 months old showed a significant shift in gut microbiota with the change from breastfeeding to solid foods (Bergstrom, Skov et al. 2014). Once established, the gut microbiota can be altered by antibiotic treatment, lifestyle, long-term change in diet, and bacterial infections (De La Cochetiere, Durand et al. 2005; Dethlefsen, Huse et al. 2008; Marques, Wall et al. 2015).
We hypothesise a link between gut microbiota and neurodevelopmental disorders via mitochondria affecting behaviour and cognition. Bacteria can affect mitochondria in several ways, for example through short-chain fatty acids (SCFAs). SCFAs are also known to affect mitochondrial function (Belzacq, Haouzi et al. 2002, Hecker, Sommer et al. 2015), for example by inducing apoptosis in colonic epithelium cells. Some bacteria are able to modulate mitochondrial function in order to maintain their living environment by preventing host cell apoptosis or to promote bacterial spread by inducing apoptosis (Matarrese, Falzano et al. 2007; Stavru, Bouillaud et al. 2011). Distressed mitochondria generate signalling molecules such as mitokines. These mitokines exit the host cell and can bind to and regulate receptors present on all eukaryotic cells. Other bacterial species, for example Pseudomonas spp. are capable of disrupting mitochondrial surveillance in Caenorhabditis elegans. Mitochondria are responsible for the synthesis of haeme and iron-sulphur clusters. Mitochondria are an attractive target for bacteria because iron is essential for bacterial processes like DNA replication and metabolism. Therefore, bacteria developed several strategies, for example production of siderophores, to acquire iron from mitochondria. Disabling the mitochondrial surveillance pathway renders other virulence factors, anti-mitochondrial toxins or siderophores more effective (Liu, Samuel et al. 2014). Pseudomonas spp. are psychrotrophic bacteria, which thrive at low temperatures (0-4 degrees Celsius). These psychrotrophic bacteria are commonly found in dairy products. We hypothesise that people ingest these bacteria more often as people tend to keep dairy products in the refrigerater more often and for longer periods of time. Finally, bacteria can affect host health and neurodevelopment through short-chain fatty acids. These SCFAs (butyrate, acetate and propionate), produced mainly by gut bacteria, are absorbed by the intestinal epithelium. SCFAs are processed by the citric acid cycle in mitochondria and used in several processes. Butyrate is used by colonocytes as source of energy. Acetate and propionate are transported via the bloodstream to other tissues and organs. Acetate is used for the synthesis of long-chain fatty acids (Christ, 1968). Propionate can be used as substrate for gluconeogenesis, a process starting in the mitochondria. Oral administration of prionate to rats led to cognitive deficits, decreased social interactions, repititive behaviour and abnormal motor activity (Shutlz *et al*., 2008). Thus, SCFAs are processed by mitochondria and fulfill several functions in the host. Dysbiosis can lead to altered levels of short-chain fatty acids (SCFA).
A major cause of gut dysbiosis is treatment with antimicrobials. Various antibiotics are potential risk factors for neurodevelopmental disorders (Atladóttir, Henriksen et al. 2012; Desbonnet, Clarke et al. 2015; Rosenfeld 2015). In addition, several antibiotic classes administered, like fluoroquinolones or aminoglycosides, are associated with mitochondrial dysfunction as a result of the close similarities between mitochondria and the targeted bacteria. This is seen as a mild side-effect and is well tolerated by most treated individuals. However, this side-effect combined with the effect of antimicrobials on the gut microbiota could possibly influence neurodevelopment, and thereby cognition and behaviour in adult life.
Another major cause of dysbiosis is diet, a key factor in determining gut microbial composition. Certain diets are able to impact cognition and behaviour to a great extent. Dietary therapies have been attempted to treat or ameliorate symptoms of a wide variety of neurological disorders, such as autism, epilepsy and Parkinson disease. In addition, dietary supplementation, for example omega-3 fatty acid or vitamin supplementation, is reported to have positive effects on symptoms of ADHD (Bos, Oranje et al. 2015; Rucklidge, Frampton et al. 2014). Obesity during pregnancy is

associated with mitochondrial dysfunction in the offspring (Wu, Russell et al. 2015) potentially leading to neurodevelopmental disorders. Childhood obesity is associated with poorer academic achievements and greater decay of brain structure and function. Obesity is also associated with reduced gut microbial diversity (Ley, Backhëd et al. 2005). Turnbaugh et al. demonstrate that these changes in diversity affect the metabolic potential of the microbiota. They show that the microbiota from obese individuals is more efficient in harvesting energy from diet compared to the microbiota from lean individuals. This efficiency in calorie harvest is transmissible from humans to mice by colonising germ-free mice with human microbiota from obese or lean individuals, indicating that dysbiosis contributes to obesity (Turnbaugh, Ley et al 2006).
There is a growing body of research reporting significant effects of mitochondrial dysfunction on brain development and function. Studies reporting associations between dysbiosis and neurodevelopment are also numerous. However, most of these studies are correlational studies, studying the correlation rather than the causality. Dysbiosis affecting mitochondrial function potentially leading to neurodevelopmental disorders has not been studied to our knowledge.

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 objective of this project is to investigate the link between dysbiosis, mitochondrial dysfunction and developmental disorders. In order to study this link we have designed a research plan composed of multiple components. The first component of this project aims to investigate the contribution of gut microbiota or specific bacteria to behaviour and cognition. The second component of this project focusses on the link between gut dysbiosis and neurodevelopmental disorders. To study this we plan to study the effects of gut dysbiosis on mitochondrial function. With the third component of this project we intend to study effects of dietary or antimicrobial treatments on gut dysbiosis, cognition and behaviour. Finally, we plan to design antimicrobial or dietary interventions to normalise gut microbial composition resulting in healthy mitochondrial function and neurodevelopment.
Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with less distress to the mice. The central theme of our research group encompasses effects of diets on neuronal systems, emphasising cognitive disorders in relation with metabolism and cerebral circulation. Important tools available include neuroimaging, including MRS and DTI, histopathology, and behavioural test equipment.
The main objective should be achievable and realistic within the duration of the project because of the availability of knowledge, expertise and accommodation.
Important publications of our research group:

- [redacted]

3.3 Relevance

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

The prevalence of many complex neurodevelopmental disorders, like ADHD and autism spectrum disorder (ASD), has been increasing across recent decades. These disorders have a huge impact on the affected individual, family members and society. Co-occurring disorders are common, such as dyslexia, obsessive compulsive disorder, depression and eating disorders. Studies have reported ADHD symptoms in 30-50% of individuals diagnosed with ASD. Similarly, around 66% of persons with ADHD show features of ASD (Davis & Kollins, 2012). Progress in understanding these disorders has been slow and treatment options are limited. In this study we will investigate the link between dysbiosis and neurodevelopmental disorders.
We will study effects of certain diets and antibiotics, which are capable of causing gut dysbiosis, on brain development and function. Investigating interventions changing gut microbial compositions and thereby influencing behaviour and cognition will increase knowledge about developmental disorders and could potentially result in new therapeutic interventions to treat or ameliorate symptoms of neurodevelopmental disorders. In addition, we aim to elucidate the link between gut dysbiosis and neurodevelopmental disorders by studying the role of mitochondrial dysfunction in these disorders.

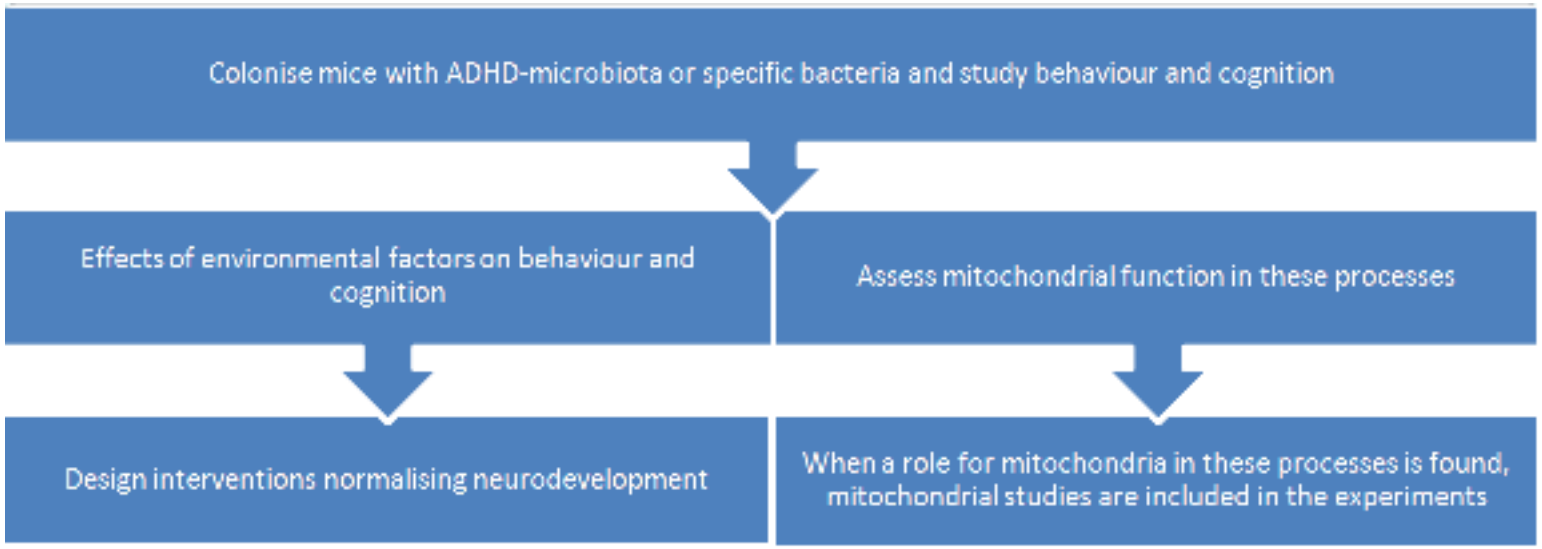
3.4 Research Strategy

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

Our main objective is to investigate the link between gut microbiota and neurodevelopment. In order to study this link we have designed several randomised experiments.

1. We aim to investigate the effects of dysbiosis on cognition and behaviour. We will do this by colonising mice with human microbiota from individuals with ADHD or by colonising mice with specific bacteria. After this we will study the impact of gut microbiota on behaviour and cognition.
2. We plan to study the link between gut dysbiosis, behaviour and function. Therefore, we will examine mitochondrial (dys)function after inducing dysbiosis.
3. We aim to study environmental factors that can lead to dysbiosis and study effects of dysbiosis on neurodevelopment.
4. We intend to design dietary and antimicrobial interventions to restore the gut microbiota to a healthy state, and thereby normalise neurodevelopment.

Overview of this project:



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.

1. Gut microbiota from people with ADHD is different in composition compared to gut microbiota from persons without ADHD (personal communication and Pärtty et al., 2015). First, we plan to study effects of the microbiota on cognition and behavior. Most gut bacteria cannot be cultured which makes studying their causal role in disorders technically challenging. To investigate whether gut dysbiosis is a cause or consequence of neurodevelopmental disorders, we will colonise germ-free mice with human microbiota from individuals with ADHD. Any dysbiosis present in the donor microbiota will be transferred to the recipient. Germ-free mice exhibit characteristics reminiscent of several neurodevelopmental disorders, like abnormal stress response and increased motor activity. This altered behaviour can be reversed by introducion gut microbiota. (Diaz Heijtz et al., 2011; Sudo et al., 2004). Thus, using conventionally raised mice, mice with normal gut microbiota, has the advantage that these mice don't show abnormal behavioural patterns and brain function. However, these mice already possess gut microbiota. When we introduce human gut microbiota in these mice, the 'new bacteria' will compete with the 'old bacteria'. Therefore, we prefer to use germ-free mice to study effects of gut microbiota op neurodevelopment. To study effects of the microbiota on neurodevelopment, we will investigate behaviour and cognition. ADHD in humans is associated with inattentiveness, hyperactivity, and/or impulsivity (American Psychiatric Association 2013). Anxiety is a very common co-morbidity in several neurodevelopmental disorders including ADHD. We can measure these characteristics in mice with behavioural tests, like the marble burying test and the open field test. Behavioural tests will be conducted to study changes in behaviour caused by gut microbiota. ADHD is not only characterised by behavioural changes but also by structural and functional brain differences (Weyandt, Swentosky et al. 2013). To cognition we will use neuroimaging

techniques, such as Diffusion Tensor Imaging (DTI), rs fMRI and Magnetic Resonance Spectroscopy (MRS), and histological and biochemical assays. After this we will compare microbial compositions from people with and without ADHD to identify suspect bacterial species for ADHD. In order to study behavioural and cognitive changes as result of specific bacteria we will colonise germ-free mice with specific bacterial species. These germ-free mice will be colonised only with these bacteria, enabling us to study behavioural and neuronal characteristics altered by these specific bacteria. After colonisation we want to examine cognition, behaviour and brain structure. When we see behavioural and/or cognitive changes as result of specific bacteria, we will infect conventionally raised mice with these bacterial species in order to mimic the natural situation.

2. Second, when we were able to induce dysbiosis by microbiota transplantation or by colonisation with specific bacteria, we plan to study the link between gut dysbiosis and neurodevelopment. To study this link we first have to induce gut dysbiosis. We will colonise mice with human ADHD-microbiota or with bacteria shown to be able to alter behaviour and cognition (first component). Inducing dysbiosis by diet or antibiotics is also possible, but inducing dysbiosis by colonising mice with microbiota or bacteria would be the best method as this will be almost instant, long-term, with only a few, mild side-effects. Changing gut microbial composition with diet takes a few months. Antibiotics directly affect mitochondria due to the striking similarities between bacteria and mitochondria, and has side-effects as well. After colonisation we will assess behaviour as well as mitochondrial function. We will also study effects of bacteria known to affect mitochondrial function, for example *Pseudomonas spp.* (Liu, Samuel et al. 2014; Manago, Becker-Flegler et al. 2015) on behaviour, cognition and mitochondrial function.

   When no altered mitochondrial function is found we will not measure mitochondrial function in future experiments.

3. Third, we aim to investigate effects of environmental factors on gut microbiota and neurodevelopment. Hereby, we focus on two environmental factors: antibiotics and diet. These two factors are associated with dysbiosis, a significant change in microbial composition. In addition, we aim to study effects of certain diets on the composition of the gut microbiota. Certain long-term diets are able to change gut microbial composition and are capable of impacting cognition and behaviour to a great extend. We plan to expose mice to these environmental factors and consequently assess behaviour and cognition using various imaging, biochemical and physiological assays. The brain is a organ with high plasticity until the end of adolecence (around 3 months). In adulthood (3-6 months) the brain is fully grown, but remains plastic. We expect therefore, that we can still ameliorate symptoms of neurodevelopmental disorders later in life.

4. If we observed effects of environmental factors in component 3, we will design specific intervention studies aiming at re-establishing a healthy microbiota. This should normalise mitochondrial energy production resulting in healthy neurodevelopment. Although diet can cause mitochondrial dysfunction via dysbiosis, these very same environmental factors, chosen carefully, could also normalise gut microbiota and mitochondrial function promoting healthy neurodevelopment. To study this we plan to give knock out mice a high fat diet. LDLr knockout

mice develop obesity when fed a high fat diet. We plan to use this mouse model to investigate effects of childhood obesity on gut microbial composition, mitochondrial function and neurodevelopment. Upon observing effects we aim to ameliorate these effects with dietary or antimicrobial interventions. The exact nature of these interventions will depend on the answers to the three above mentioned components of this project.

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

All components of this project are focussed on the link between dysbiosis and neurodevelopmental disorders such as autism and ADHD. We will address the effects of several interventions, such as diet or antimicrobials, on gut microbiota, mitochondrial function, and cognition and behaviour. These experiments will require critical timing as interventions and colonisations need to take place before finalisation of neurodevelopment.
To study this we formulated the following milestones:

1. Gut microbiota has an impact on neurodevelopment and we can identify suspect bacterial species affecting cognition and behaviour.
   If we observe affected neurodevelopment as result of the gut microbiota, we will explore the role of mitochondria in this process.
2. *Dysbiosis leads to mitochondrial dysfunction.*
   If we were able to demonstrate affected mitochondrial function in these processes, we will also assess mitochondrial function after dietary interventions. If we can show effects of gut microbiota and/or specific bacteria on behaviour and cognition, we will explore effects of diets or antibiotic treatment on gut microbial composition and neurodevelopment.
3. Environmental factors, such as diet and/or antibiotics, lead to gut dysbiosis and consequently affect neurodevelopment.
   When we observe effects of environmental factors on dysbiosis and on neurodevelopment, we will design dietary interventions to re-establish a healthy gut microbiota, leading to healthy neurodevelopment.
4. Mitochondrial energy production and neurodevelopment can be normalised by dietary interventions.

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	Effects of microbiota or specific bacteria on cognition and behaviour
2	Effects of antibiotics on gut microbiota and neurodevelopment
3	Effects of diet on gut microbiota and neurodevelopment
4	Interventions to re-establish a healthy gut microbiota

12.

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.zbo-ccd.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 Effects of microbiota or specific bacteria on cognition and behaviour

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.

In order to study effects of the gut microbiota on neurodevelopment we plan to colonise mice with specific bacterial species or with human microbiota from individuals diagnosed with ADHD. After colonisation we aim to conduct behavioural tests followed by measuring mitochondrial function or performing neuroimaging tests. The general design for this experiment is as follows:

1. Colonisation

2. Behavioural tests

3. Measuring mitochondrial function or conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure post mortem brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.

All experiments, except neuroimaging and measuring mitochondrial function, will be performed in isolators wherein the mice are housed to prevent colonisation with environmental bacteria. As we house mice in isolators we are restricted in options for behavioural tests. This excludes all equipment requiring power. In addition, we cannot use equipment too large to fit inside the isolators (larger than 45x45cm).

We will colonise mice with microbiota or specific bacteria. We will collect stool samples once a week and sequence bacterial 16S rRNA genes. We will analyse stability of colonisation by comparing the taxonomic profiles of stool samples with the original sample.
Before and after colonisation we will conduct behavioural tests (at most once a week) in order to study behavioural changes after colonisation. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory.

After behavioural tests, mice are randomly divided into two groups: one group will be used for analysis of brain structure and function while the other group will be used to study post mortem brain mitochondrial function. Isoflurane, used to anaesthetise mice during neuroimaging, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.
Mice used for neuroimaging techniques are sacrificed directly after scanning by and their brains will be used to examine brain structure using histological and biochemical assays.

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

Before we start with this project, we will practise techniques, such as gavage, neuroimaging, transcardial perfusion, and measuring mitochondrial function, on surplus mice to optimise succes rate of these experiments.
This experiment will take maximally 4 weeks. The aim of this experiment is to study effects of the gut microbiota on neurodevelopment. We will first colonise mice with human microbiota or with specific bacteria to study whether gut dysbiosis is a cause of neurodevelopmental disorders. Any dysbiosis present in the donor microbiota will be transferred to the recipient mouse. Most of the bacteria present in gut microbiota cannot be cultured, this makes studying their causal role in disorders technically challenging. After colonisation we will conduct behavioural tests and analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders.
This approach allows us to study effects of gut microbiota on neurodevelopmental disorders. First, wel will colonise germ-free mice with human microbiota of specific bacteria. After that, we will study behaviour and cognition to investigate the causality between gut dysbiosis and neurodevelopmental disorders.
All mice will be colonised with a suspension of microbiota or specific bacteria in Phosphate Buffered Saline (PBS) via gavage. By oral force-feeding we can decrease variation as every mouse will receive exactly the same amount of microbiota. Administration of bacterial suspensions via gavage will have a mild, short-term impact on the animals. This procedure will be done once every week in order to reinforce the microbiota and will take about 5 seconds per mouse.
Behavioural tests can possibly cause mild, short-term distress. Tests conducted include the Open Field test, marble burying test, and object recognition test. These tests will be done at most once a week (before and after colonisation) in all mice and will take about 10 minutes each time. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory.
Brain function and structure, for example investigating cerebral blood flow, connectivity structures, or grey and white matter integrity, will be examined in one group of mice. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be sacrificed directly after these MRI experiments by transcardial perfusion-fixation using Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial

perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.Mitochondrial function will be measured in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed under comparable conditions. Introducing microbiota via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies.
We will first investigate if we are able to alter behaviour and cognition by microbiota transplantation or colonisation with bacteria. When we see behavioural and cognitive changes, we will include a group of mice to measure mitochondrial function. By practising challenging techniques on surplus animals, we will minimise the number of mice required for these experiments.

B. The animals

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

We will use germ-free as well as conventionally raised mice (Mus musculus). Germ-free mice, mice without microbiota, are required for colonisation. These mice have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice (3 weeks old), after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala.
Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females. Before we start this project we will practise the required skills with surplus mice. These skills include gavage, MRI scanning and measuring mitochondrial function. The maximum number of animals we consider to be necessary to practise is 40 mice in total in this project.
We will use test groups (N=14), mice that receive human microbiota or specific bacteria, as well as control groups (N=14), mice receiving a sham treatment. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. The maximum number of animals we consider to be necessary is 224 mice, allowing us to conduct 8 experiments to analyse altered behaviour, brain function, and brain structure as result of microbiota or specific bacterial species. With these 8 experiments we plan to do the following procedures:

- 1 experiment to investigate whether ADHD gut microbiota affects behaviour and cognition in germ-free mice
- 4 experiments to analyse effects of specific bacterial species on behaviour and cognition in germ-free mice
  followed by:
- 3 eperiments to analyse effects of specific bacteria (selected in germ-free mice) species on behaviour and cognition in conventionally raised mice.

The specific bacterial species used will include Pseudomonas spp., often found in dairy products, and Lactobacillus and Bifidobacterium, often used in probiotics.
When behaviour and cognition are altered after colonisation, we will also include a group of mice to assess mitochondrial function (N=10).
Mitochondrial function will be assessed in mice colonised with the microbiota or bacteria shown to be most effective in changing behavioural and cognition. An additional 40 mice is required when mitochondrial function is proven to be affected by dysbiosis.
The total number of animals we consider necessary is 264 mice for this experiment and 40 mice to practise required skills.

Species	Origin	Maximum number of animals	Life stage
Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	304	3 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:
  We plan to investigate effects of gut microbiota or specific bacterial species on behaviour, cognition and mitochondrial function.
  The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.

- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. We will only assess mitochondrial function when we were able to affect behaviour and cognition by colonisation. Mitochondrial function is studied only for microbiota or bacteria shown to be most effective in changing behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. We will either colonise mice with microbiota or introduce specific bacteria. Using one of these methods, introduced gut dysbiosis will be almost instant, long-term and with least side-effects. Inducing dysbiosis with diet will take three months and using antibiotics to promote gut dysbiosis will also introduce side effects. After inducing intestinal dysbiosis we will conduct behavioural tests to assess behavioural changes due to the dysbiosis. The used behavioural tests give as little as possible distress and pain, while still yielding reliable results. When we see behavioural changes in the animals we will assess cognition in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

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

G. Location where the animals procedures are performed

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

[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, analgesiaor 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?

Negative effects from colonisation with bacteria, mostly gastrointestinal problems like diarrhoea, may be expected. Behavioural and cognitive changes caused by the colonisation such as hyperactivity and increased anxiety. We know from experience that distress caused by these changes is mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Effects of the microbiota or bacteria on behaviour and cognition are described in literature. Some of the bacteria are known to occasionally cause diarrhoea in humans. Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them. Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

Transplanting human microbiota to germ-free mice has been done before (Ridaura, Faith et al. 2013; Bruce-Keller, Salbaum et al. 2015) where the researchers studied the role of gut microbiota on obesity. No adverse effects, except a significant increase in weight, were reported. Our goal is to study effects of microbiota from individuals with ADHD on cognition and behaviour in mice. Distress caused by the expected behavioural and cognitive changes is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 1%.

K. Classification of severity of procedures

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

The discomfort caused by the colonisation can be classified as mild to moderate. Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category ‘non-recovery’.
Cumulative levels of discomfort are expected to be mild (80%) to moderate (20%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed by cervical dislocation in order to study brain mitochondrial function and structure. The other group of mice will be sacrificed in order to study brain function and structure.

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

[] No > Descripe 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.zbo-ccd.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 Effects of antibiotics on gut microbiota and neurodevelopment

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.

In order to investigate effects of antibiotics on dysbiosis, mitochondrial function and brain development, we will expose mice to these antibiotic-treatments. Some antimicrobial classes are associated with gut dysbiosis and mitochondrial dysfunction. We will use often described antibiotics which possibly affect mitochondrial function, such as fluoroquinolones and aminoglycocides. In order to study effects of environmental effects on gut microbial composition, we will collect stool samples once a week.
After treatment we aim to conduct behavioural tests followed by measuring mitochondrial function or performing neuroimaging tests. The general design for this experiment is as follows:

1. Treatment of conventionally raised mice with antibiotics

2. Behavioural tests

3. Measuring mitochondrial function or conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure *ex vivo* brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.
Before and after treatment with antibiotics we will conduct behavioural tests in order to study behavioural changes after colonisation. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory.
After behavioural tests, mice are randomly divided into two groups: one group will be used for analysis of brain structure and function while the other group will be used to study brain mitochondrial function. Isoflurane, used to anaesthetise mice during neuroimaging, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.

Mice used for neuroimaging techniques are sacrificed directly after scanning and their brains will be used to examine brain structure using histological and biochemical assays.

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

This experiment will take maximally 5 weeks. The aim of this experiment is to study effects of antibiotics on gut dysbiosis and neurodevelopment. We will used conventionally raised mice, mice with normal gut microbiota. We will administer antibiotics via drinking water. We will select antibiotics known to affect mitochondrial function and often administered. To investigate effects of antimicribials on neurodevelopment, we will conduct behavioural tests and analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders.
This approach allows us to study effects of antibiotics on neurodevelopmental disorders. We will administer antibiotics to mice with normal gut microbiota. After that, we will study behaviour and cognition to investigate whether antibiotic-treatments affects neurodevelopment

All mice will be treated with antibiotics, such as fluoroquinolones, via drinking water. We will introduce these antimicrobial interventions to induce gut dysbiosis and after one month on antibiotic-treatment we will examine behaviour, brain structure, and brain function with behavioural studies and neuroimaging techniques. Every week (before and after treatment) stool samples will be collected to analyse microbial composition.
Behavioural tests, for example the Open Field test, the Marble Burying test, and the object recognition test, can possibly cause mild, short-term distress. These tests will be done at most once a week (before and after colonisation) in all mice and will take about 10 minutes each time. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory.
Brain function and structure, for example investigating cerebral blood flow, connectivity structures, or grey and white matter integrity, will be examined in one group of mice. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be sacrificed directly after these MRI experiments by transcardial perfusion-fixation using 1 M Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.
Mitochondrial function will be measured in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed in comparable conditions. Introducing microbiota via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. All experiments are performed by experienced researchers or technicians.
We will carefully select potential antibiotics in order to minimise the groups of mice needed for this experiment. Mitochondrial function will be measured only when we were able to show altered mitochondrial function in the previous experiment (animal procedure 1), or when we see behavioural and/or cognitive changes after antibiotica-treatment.

B. The animals

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

We will use conventionally raised mice (Mus musculus). These conventionally raised mice, mice with normal microbiota, have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice, after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala. Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females.
G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. We will use test groups (N=14), mice that receive antibiotics associated with mitochondrial dysfunction, as well as control groups (N=14). The maximum number of animals we consider to be necessary is 140 mice, allowing us to conduct 5 experiments to analyse altered behaviour, brain function, and brain structure as result of treatment with antibiotics. With these 5 experiments we plan to study effects of five selected classes of antibiotics, including aminoglycosides, beta-lactam, chloramphenicol, fluoroquinolones and oxazolidinones. We start with three of these classes that are most often administrered to young children or pregnant women. When we don't see effects of these three classes of antibiotics we will end this experiment.
When behaviour and cognition are altered after antibiotic-treatment, we will also use a group of mice to assess mitochondrial function (N=10). Mitochondrial function will be assessed in mice treated with antibiotics shown to be most effective in changing behavioural and cognition. An additional 40 mice is required when behavioural and cognition are proven to be affected by dysbiosis. The total number of animals we consider necessary is 180 mice for this experiment.

Species	Origin	Maximum number of animals	Life stage

Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	180	3 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:
  We plan to investigate effects of gut dysbiosis on behaviour, cognition and mitochondrial function. The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.

- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. We will only assess mitochondrial function when we were able to affect behaviour and cognition by antibiotics or when mitochondrial function is shown to be affection in animal procedure 1. Mitochondrial

function is studied only for antibiotics shown to be most effective in changing behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. We will administer mice antibiotics associated with affected neurodevelopment. The used behavioural tests give as little as possible distress and pain, while still yielding reliable results. When we see behavioural changes in the animals we will assess cognition in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane. Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

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

G. Location where the animals procedures are performed

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

[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, analgesiaor 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?

Mild side-effects from antibiotic treatment, mostly gastrointestinal problems like diarrhoea, may be expected. Behavioural and cognitive changes caused by the antibiotics such as hyperactivity and increased anxiety. We know from experience that distress caused by these changes is mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Antibacterials commonly cause some side-effects as antibiotics can destroy commensal bacteria living in the host. Our goal is to study effects of diet or usage of antibiotics on cognition and behaviour in mice. We expect to see behavioural and cognitive changes. Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them.Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

Occurrence of side-effects from antibiotics cannot be prevented. These potential effects are considered to be mild with no significant impairment of the well-being or general condition. Humane endpoints are adopted to minimise severity. Distress caused by the expected behavioural and cognitive changes is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 5%.

K. Classification of severity of procedures

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

The discomfort caused by the antibiotics can be classified as mild. Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category 'non-recovery'. Cumulative levels of discomfort are expected to be mild (100%).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed by cervical dislocation in order to study brain mitochondrial function and structure. The other group of mice will be sacrificed in order to study brain function and structure.

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

[] No > Descripe 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.zbo-ccd.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 3	Type of animal procedure Effects of diet on gut microbiota and neurodevelopment

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.

In order to investigate effects of diet on dysbiosis, mitochondrial function and brain development, we will expose mice to these dietary interventions. Certain diets are able to impact cognition and behaviour to a great extent. Diets selected in this experiment are associated with neurodevelopment, for example high fat or high sugar diets. In addition, we will also study effect of diets considered healthy like diets high in omega-3 or carbohydrates. In order to study effects of diet on gut microbial composition, we will collect stool samples once a week.
After treatment we aim to conduct behavioural tests followed by measuring mitochondrial function or performing neuroimaging tests. The general design for this experiment is as follows:

1. Diet conventionally raised mice

2. Behavioural tests

3. Measuring mitochondrial function or conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure *ex vivo* brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.

Before and after treatment with diets we will conduct behavioural tests in order to study behavioural changes after colonisation. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory.
After behavioural tests, mice are randomly divided into two groups: one group will be used for analysis of brain structure and function while the other group will be used to study brain mitochondrial function. Isoflurane, used to anaesthetise mice during neuroimaging, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.

Mice used for neuroimaging techniques are sacrificed directly after scanning and their brains will be used to examine brain structure using histological and biochemical assays.

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

This experiment will take maximally 3 months. The aim of this experiment is to study effects of diet on gut dysbiosis and neurodevelopment. We will used conventionally raised mice, mice with normal gut microbiota. We will give mice diets associated with neurodevelopment, for example high fat or high sugar diets. To investigate effects of diet on neurodevelopment, we will conduct behavioural tests and analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders.
This approach allows us to study effects of diet on neurodevelopmental disorders. We will give mice diet and after three months we will study behaviour and cognition to investigate whether diet affect neurodevelopment
All mice will be fed special diets, for example high saturated fat and high caloric/sucrose diets or diets with unsaturated fatty acids. We will introduce these dietary interventions to induce gut dysbiosis and after three months on the diets we will examine behaviour, brain structure, and brain function with behavioural studies and neuroimaging techniques. Every week (before and after treatment) stool samples will be collected to analyse microbial composition.
Behavioural tests can possibly cause mild, short-term distress. Tests conducted include the Open Field test, marble burying test, and object recognition test. These tests will be done at most once a week (before and after colonisation) in all mice and will take about 10 minutes each time. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory.
Brain function and structure, for example investigating cerebral blood flow, connectivity structures, or grey and white matter integrity, will be examined in one group of mice. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be sacrificed directly after these MRI experiments by transcardial perfusion-fixation using 1 M Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.Mitochondrial function will be measured in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed in comparable conditions. Introducing microbiota

via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. All experiments are performed by experienced researchers or technicians.
We will carefully select potential diets in order to minimise the groups of mice required for this experiment. Mitochondrial function will be measured only when we were able to show altered mitochondrial function in the previous experiment (animal procedure 1), or when we see behavioural and/or cognitive changes after diets.

B. The animals

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

We will use conventionally raised mice (Mus musculus). These conventionally raised mice, mice with normal microbiota, have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice, after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala. Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females.
G*Power is used to determine the appropriate sample size for these tests (P<0.05; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. We will use test groups (N=14), mice that receive diets associated witn neurodevelopmental disorders, as well as control groups (N=14), mice given healthy diets. The maximum number of animals we consider to be necessary is 112 mice, allowing us to conduct 4 experiments to analyse altered behaviour, brain function, and brain structure as result of diet. With these four experiments we plan to study effects of four selected diets, including diets with high saturated or unsaturated fatty acids and high calorie/sugar diets.
When behaviour and cognition are altered after diet, we will also use a group of mice to assess mitochondrial function (N=10). Mitochondrial function will be assessed in mice given diets shown to be most effective in changing behavioural and cognition. An additional 40 mice is required when behavioural and cognition are proven to be affected by dysbiosis. The total number of animals we consider necessary is 152 mice for this experiment.

Species	Origin	Maximum number of animals	Life stage
Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	152	3 weeks

C. Re-use

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:
  We plan to investigate effects of gut dysbiosis on behaviour, cognition and mitochondrial function. The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.

- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. We will only assess mitochondrial function when we were able to affect behaviour and cognition by diet or when mitochondrial function is shown to be affection in animal procedure 1. Mitochondrial function is studied only for antibiotics shown to be most effective in changing behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. We will give mice diets associated with affected neurodevelopment. The used behavioural tests give as little as possible distress and pain, while still yielding reliable

results. When we see behavioural changes in the animals we will assess cognition in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

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

F. Accommodation and care

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, analgesiaor 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?

Some diets can cause mild distress. Mostly gastrointestinal problems like diarrhoea, may be expected. Behavioural and cognitive changes caused by the induced dysbiosis such as hyperactivity and increased anxiety. We know from experience that distress caused by these changes is mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Feeding mice modified diets, that do not meet all of the nutritional needs are expected to cause mild distress. Our goal is to study effects of diet on cognition and behaviour in mice. We expect to see behavioural and cognitive changes. Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them.Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

We will prefer to use diets that do meet all of the animals' nutritional needs to prevent distress. Potential effects from diets are considered to be mild with no significant impairment of the well-being or general condition within the time-scale of this study. Humane endpoints are adopted to minimise severity. Distress caused by the expected behavioural and cognitive changes is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 1%.

K. Classification of severity of procedures

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

The discomfort caused by the diets can be classified as mild. Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category 'non-recovery'.
Cumulative levels of discomfort are expected to be mild (100%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed by cervical dislocation in order to study brain mitochondrial function and structure. The other group of mice will be sacrificed in order to study brain function and structure.

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

[] No > Descripe 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.zbo-ccd.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 4	Type of animal procedure Interventions to re-establish a healthy gut microbiota

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.

We will carry out this experiment only when we see changes in behaviour and cognition after introducing antibiotics, diet (previous described animal procedures).
Obesity during pregnancy is associated with mitochondrial dysfunction in oocytes. Childhood obesity is associated with poorer academic achievements and greater decay of brain structure and function. Certain knock-out mice, for example the LDLr-/- mice, develop obesity after fed a high fat diet. We plan to use these knock-out mice to investigate effects of childhood obesity on gut microbial composition, mitochondrial function and brain function. The brain is an organ with high plasticity. Therefore we plan to design dietary intervention studies to re-establish a healthy gut microbiota, thereby normalising mitochondrial energy production resulting in healthy neurodevelopment.When we observe behavioural or cognitive effects of obesity we will aim to ameliorate these effects using dietary or antimicrobial interventions to re-establish a healthy gut microbiota, thereby normalising mitochondrial energy production resulting in healthy neurodevelopment. The exact nature of these interventions largely depends on outcomes of previous experiments. The general design for this experiment is as follows:

1. Introducing intestinal dysbiosis by high-fat diet
2. Behavioural tests
3. Treatment of gut dysbiosis either by diet, antibiotics or probiotics(animal procedure 3)
4. Behavioural tests
5. Measuring mitochondrial function and conducting neuroimaging techniques

Neurodevelopmental disorders are disabilities in which the development of the central nervous system is disturbed. Our primary outcome parameters are behaviour and cognition. These are characteristics typically affected in neurodevelopmental disorders. Behaviour and cognition will be assessed by behavioural tests, neuroimaging techniques, histology, and biochemical assays. We will measure *ex vivo* brain mitochondrial function to explore whether mitochondria play a role in neurodevelopment.
We will first feed LDLr-/- mice a high fat diet to induce obesity. After this, we will collect stool samples to analyse microbial composition and conduct behavioural tests to investigate effects of obesity on behaviour. We will measure behaviour like motor activity and anxiety using the Open Field test, a large square chamber. Typically, mice will spend a greater amount of time exploring the periphery of the Open Field test rather than the unprotected centre area. Mice that spend more time exploring the centre area show anxiety-like behaviour. The marble burying test is often used to assess characteristics of several neurodevelopmental disorders, including anxiety and obsessive-compulsive behaviour. Mice with a high degree of anxiety or repetitive behaviour tend to engage in a high degree of digging. Thus, the greater number of burried marbles is an indication of heightened anxiety-like or repetitive behaviour, often seen in people with neurodevelopmental disorders. The object recognition test is used to test recognition memory. During the first session, mice are presented two identical objects. After this session, one of the objects is replaced with a different object. The amount of time spend to explore the new object provides an index of recognition memory. When we see changes in behaviour,

we will treat these mice with dietary of microbial interventions. The exact nature of these interventions used will depend on the results of animal procedure 3.After dietary intervention, we will conduct behavioural tests to see if the diet is able to ameliorate changes in behaviour.
We will focus on brain structure and function first. When we see changes in behaviour, brain structure, and brain function as result of one or more treatments, we will study mitochondrial function as well. Isoflurane, used to anaesthetise mice during neuroimaging to study brain function, is known to affect mitochondrial function. Therefore, it would be unreliable to measure mitochondrial function in isoflurane-treated mice. A separate group of mice is required to measure mitochondrial function. At the end of these experiments, we will correlate behaviour to brain structure and function, as well as correlate behaviour to mitochondrial function and structure. For this reason we want to test behaviour in all mice.
Mice used for neuroimaging techniques are sacrificed directly after scanning and their brains will be used to examine brain structure using histological and biochemical assays.

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

This experiment will take maximally 4 months. The aim of this experiment is to design dietary interventions able to normalise gut microbial composition and neurodevelopment. To study possible interventions, we first have to induce dysbiosis. We will do this by first feed LDLr-/- mice a high fat diet to induce obesity and dysbiosis. After this we will conduct behavioural tests to study alterations in behaviour caused by dysbiosis. We then will give mice diets selected in animal procedure 3. We will again conduct behavioural tests to see if these diets are able to modify behaviour. Finally we will analyse brain function and structure. Behaviour and cognition are traits affected in persons with neurodevelopmental disorders. This approach allows us to study dietary or antimicrobial interventions to ameliorate traits of neurodevelopmental disorders. First, neurodevelopmental disorders are mimiced by inducing dysbiosis leading to altered behaviour and cognition. We then try to ameliorate these symptoms by giving mice high potential diets (selected in the third animal procedure). In order to design interventions to ameliorate effects of the gut dysbiosis on neurodevelopment and function we first aim to induce gut dysbiosis in LDLr-/- mice. After inducing dysbiosis, we will conduct behavioural tests (before and after inducing dysbiosis) which will take about 10 minutes each time. Behavioural tests can possibly cause mild, short-term distress. Tests conducted include the Open Field test, marble burying test, and object recognition test. These tests will be used to study behaviour, such as anxiety, attention, locomotor activity and self-grooming. ADHD is characterised by attention difficulties, impulsivity and hyperactivity. Hyperactivity and anxiety are often studied using the Open Field test. The Marble Burying test can be used to assess repetitive, compulsive-like behaviour, commonly seen in individuals diagnosed with neurodevelopmental disorders, such as autism and ADHD. The object recognition test can be used to assess recognition memory. When we see behavioural changes, we will treat mice with dietary interventions to ameliorate those symptoms using diets. The exact nature of the diet is dependent on outcomes of previous experiments (animal procedure 3). For example, we will give diets containing high saturated fat or unsaturated fatty acids, high caloric/sucrose, or diets containing probiotics. Mice will be fed diets ad libitum for approximately 3 months. During dietary intervention, we will conduct behavioural tests once a week to assess behavioural changes. We will use the same behavioural tests described above.
Three months after starting on the diet, we will investigate brain function and structure in one group of mice. For this, we will examine cerebral blood flow, connectivity structures, or grey and white matter integrity. Mice will be anaesthetised using 1.5% isoflurane via inhalation. During the MRI experiments heart rate and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with a warm air flow. Analysis of brain structure and function will be done under general anaesthesia from which the animals do not recover consciousness. Mice will be

sacrificed directly after these MRI experiments by transcardial perfusion-fixation using Phosphate Buffered Saline (PBS) while the mice are still under general anaesthesia. Transcardial perfusion-fixation will be used as killing method to remove blood from the brain. After mice are sacrificed, brains will be used for histological and biochemical analysis.
Only when we have seen altered mitochondrial function as result of dysbiosis (animal procedure 1-3) we will examine mitochondrial function in this experiment. In order to study whether dietary interventions are able to normalise brain mitochondrial function, we will measure mitochondrial function in the other group of mice directly after sacrificing the mice by cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as respiration, complex I-IV activity, mitochondrial membrane potential and ATP/ADP levels.

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

Several considerations have been taken into account in order to minimise the number of animals. We will use littermate male mice of same weight. Mice are randomly placed in test or control groups. Both groups will be treated similarly and housed in comparable conditions. Introducing microbiota via gavage will also decrease variation. G*Power is used to determine the appropriate sample size for these tests ($P<0.05$; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. All experiments are performed by experienced researchers or technicians.
Mitochondrial function will be measured only when mitochondrial function is proven to be affected by dysbiosis in previous experiments (animal procedure 1-3). Measuring mitochondrial function will only be performed in mice given the diet with the highest potential in affecting behaviour and cognition.

B. The animals

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

We will use germ-free mice (Mus musculus). Germ-free mice, mice without microbiota, are required for colonisation. These mice have been widely used in studies investigating the effects of the gut microbiota on host brain function and development. Manipulations of the gut microbiota, such as antibiotic treatment, colonising germ-free mice and dietary interventions, are widely investigated and characterised in mice. Availability of germ-free and specific knock-out mice are indispensible because they allow manipulations that cannot be done in humans to study influences of the gut microbiota on brain structure and function. We plan to treat young mice, after weaning, before completion of the neurodevelopment. This is well before onset of adolescence (around 6-7 weeks). Before and during early puberty, an overproduction of axons and synapses occurs, followed by rapid pruning in later adolescence. Into early adulthood (at around 3 months), there is a continual growth in density of the fibers connecting the prefrontal cortex and the amygdala. Mice are subjected to a treatment for at least one month before neuroimaging tests will be performed. We intend to use male mice because many features of neurodevelopmental disorders, for example hyperactivity and repetitive behaviours, are more pronounced in males compared to females.
G*Power is used to determine the appropriate sample size for these tests ($P<0.05$; Power: 0.80). Effect sizes and anticipated standard deviations are based on prior comparable studies. The number of groups of mice used for this experiment depends on how many diets show potential in animal

procedure 4. We will start with the diets showing highest potential in affecting behaviour and cognition. We will use test groups (N=14), as well as control groups (N=14). The maximum number of animals we consider to be necessary is 112 mice, allowing us to conduct 4 experiments to analyse altered behaviour, brain function, and brain structure. When mitochondrial function is altered in previous experiments, we will also use a group of mice to assess mitochondrial function (N=10). Measuring mitochondrial function will only be performed in mice given the diet with the highest potential in affecting behaviour and cognition. An additional 20 mice is required when mitochondrial function is proven to be affected by dysbiosis. The total number of animals we consider necessary is 132 mice for this experiment.

Species	Origin	Maximum number of animals	Life stage
Mice (Mus musculus)	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	132	3 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:
  We plan to investigate effects of gut dysbiosis on behaviour, cognition and mitochondrial function. The pathways from gut to brain are complex and not yet fully understood. It is impossible to accurately mimic behaviour and cognition in vitro, thus replacing animals with in vitro experiments is not an option. As neurodevelopmental disorders are very complex, substituting mice with lower animals, such as

zebrafish, is not possible. Mitochondrial function will be measured ex vivo as well as analysis of brain structure. Substituting behavioural experiments by in vitro or ex vivo experiments is not possible.

- Reduction:
  We took several considerations into account to minimise variations between animals and thereby minimising the total number of required animals. We calculated minimum required numbers of mice using G*Power. This experiment is only carried out when previous experiments (animal procedures 2 and 3) were successful. We will only assess diets that were proven to be able to affect behaviour and cognition in animal procedure 4. Diets with the highest potentials are investigated first. We will only measure mitochondrial function in these mice when we demonstrated altered mitochondrial function in previous experiments. For mitochondrial function, we will only assess the diet most affecting behaviour and cognition.

- Refinement:
  Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. We will conduct behavioural tests to assess behavioural changes due to the dysbiosis. The used behavioural tests give as little as possible distress and pain, while still yielding reliable results. When we see behavioural changes in the animals we will try to ameliorate these changes by treating the mice with dietary interventions. These interventions are chosen carefully in animal procedure 3. A group of control mice is given normal chow. Changes in behavioural are measured by the same behavioural tests used before the treatment. Finally cognition is assesed in both test and control mice using neuroimaging techniques to analyse brain physiology. Cerebral blood flow, connectivity structures, or grey and white matter integrity are commonly affected in individuals with neurodevelopmental disorders. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Humane endpoints are implemented to prevent further distress. Mitochondrial function will only be studied when proven to be able to affect behaviour and cognition in previous experiments. Mice are sacrificed by cervical dislocation because isoflurane and CO2 are known to affect mitochondrial function.

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

Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. Pain and distress during MRI experiments will be reduced by anaesthetising the mice by means of inhalation of 1.5% isoflurane (Protocol CDL, Nijmegen). Analgesia during experiments will not be necessary. To minimise pain and distress, humane endpoints are implemented. After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed. Expertise on the proposed experiments is available as well as adequate equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least distress to the mice.

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.

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?

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

G. Location where the animals procedures are performed

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

[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, analgesiaor 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?

Distress caused by behavioural changes are considered to be mild. During neuroimaging tests, mice are anaesthetised using 1.5% isoflurane. Effects of isoflurane include respiratory depression and a decrease in blood pressure. The level of anaesthesia can be adjusted rapidly. Mice used to assess mitochondrial function are sacrificed immediately by cervical dislocation. No other adverse effects on animal welfare will be expected

Explain why these effects may emerge.

Some behavioural tests, such as the Open Field test, can cause mild distress because mice are placed in an area unfamiliar to them. Anaesthetic-induced respiratory depression is not an uncommon effect of anaesthesia.

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

Distress caused by the expected behavioural and cognitive changes as result of obesity is considered mild. We will introduce animals to the behavioural tests before testing in order to lower stress levels. Heart rate and body temperature will be closely monitored and supported during isoflurane-treatment

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.

Debilitating diarrhea, rapid weight loss, dehydration. Bleeding from any orifice, edema, self-induced trauma, any condition interfering with daily activities (for example, eating or drinking), excessive or prolonged hypothermia or hyperthermia.
After reaching the implemented humane endpoints, we will consult the veterinarian or animal welfare expert in order to determine whether the animals should be sacrificed.

Indicate the likely incidence.

The incidence of these humane endpoints is unlikely. Number of animals to suffer from debilitating diarrhoea is expected to be less than 1%.

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).

Levels of discomfort as result of behavioural tests are expected to be mild. Animals to be sacrificed to measure brain mitochondrial function are killed via cervical dislocation. Levels of discomfort are classified as non-recovery. Mice used to assess brain structure are anaesthetised and will not recover consciousness. This procedure is assigned to category ‘non-recovery’.
Cumulative levels of discomfort are expected to be mild (80%) to moderate (20%)

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.
[X] Yes > Explain why it is necessary to kill the animals during or after the procedures.

After finishing the experiment the mice will be sacrificed in order to study brain function and structure, or brain mitochondrial function and structure.

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

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

[X] Yes

1□

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Onze referentie
Aanvraagnummer
AVD103002015208

Uw referentie

Bijlagen
1

Datum 28-8-2015
Betreft Beslissing Aanvraag projectvergunning dierproeven

Geachte heer/mevrouw,

Op 04 augustus 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'effecten van de darm microbiota op ontwikkelingsstoornissen' met aanvraagnummer AVD103002015208. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag gedeeltelijk goed op grond van artikel 10a van de Wet op de dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. Alleen proef 1 en 2 van het project kunnen vergund worden omdat er nog te veel onzekerheden zijn over proef 3 en 4. De uitkomsten van proef 1 en 2 zijn bepalend voor niet alleen de wetenschappelijke validiteit, maar ook de inrichting van proef 3 en 4.
U kunt met uw project "effecten van de darm microbiota op ontwikkelingsstoornissen" starten. De vergunning wordt conform uw aanvraag afgegeven van 8 september 2015 tot en met 8 september 2019.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie RUDEC gevoegd. Dit advies is opgesteld op 07 augustus 2015. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a lid 3 van de wet. Wij kunnen ons niet geheel vinden in de inhoud van het advies van de Dierexperimentencommissie. Over de wetenschappelijke validiteit en de inrichting van proeven 3 en 4 zijn nog te veel onzekerheden. Nut en noodzaak, alsmede de inrichting van deze proeven worden pas duidelijk na afloop van proef 1 en 2. Wij nemen het advies van de dierexperimentencommissie daarom deels over, inclusief de daaraan ten grondslag liggende motivering.
Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

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

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

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

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

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

Met vriendelijke groet,

De Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

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

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

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan
Naam: Stichting Katholieke Universiteit Nijmegen
Adres: Postbus 9102
Postcode en woonplaats: 6525 EZ NIJMEGEN
Deelnemersnummer: 10300

deze projectvergunning voor het tijdvak 08 september 2015 tot en met 08 september 2019, voor het project 'effecten van de darm microbiota op ontwikkelingsstoornissen' met aanvraagnummer AVD103002015208, volgens advies van Dierexperimentencommissie RUDEC.

Hierbij is afgeweken van het DEC-advies omdat er over proef 3 en 4 nog te veel onzekerheden bestaan. Niet alleen de wetenschappelijke validiteit maar ook de inrichting van proef 3 en 4 is afhankelijk van de uitkomst van proef 1 en 2.
De functie van de verantwoordelijk onderzoeker is [redacted].
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen op 04 augustus 2015.
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 04 augustus 2015;
   b. Niet-technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 04 augustus 2015;
   c. Advies van Dierexperimentencommissie, ontvangen op 04 augustus 2015
   d. De aanvullingen op uw aanvraag, ontvangen op 20 augustus 2015.

Dierproeven

Naam dierproef	Diersoort	Aantal dieren	Ernst	Voorwaarden
Proef 1	Muis	304	80% licht, 20% matig	Zie onder voorwaarden
Proef 2	Muis	180	80% licht, 20% matig	Zie onder voorwaarden

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

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

De CCD is verder van mening dat het doen van het geplande onderzoek in een speciale germ-free omgeving zeer moeilijk uitvoerbaar is vanwege de beperkte ruimte. Dit behoeft extra aandacht van de IVD. Daarom adviseert de CCD dat de experimenten op praktische uitvoerbaarheid getoetst worden door de IVD.

Weergave wet- en regelgeving

Dit project en wijzigingen

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

Verzorging

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

Pijnbestrijding en verdoving

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

Einde van een dierproef

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

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