	Inventaris Wob-verzoek W16-18S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2016481								
1	Aanvraagformulier				x		x		
2	Projectvoorstel oud			x					
3	Bijlage beschrijving dierproeven 1, 2, 3 en 4 oud			x					
4	Niet technische samenvatting oud			x					
5	DEC-advies				x		x		
6	Ontvangstbevestiging				x		x		
7	Verzoek aanvulling aanvraag				x		x		
8	Reactie verzoek aanvulling				x		x		
9	Projectvoorstel nieuw			x					
10	Bijlage beschrijving dierproeven 1, 2, 3 en 4 nieuw			x					
11	Niet technische samenvatting nieuw	x		x					
12	Adviesnota CCD		x						x
13	Beschikking en vergunning				x		x		

AVD 1030020164 81

22 MAART 2016

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☑ Ja > *Vul uw deelnemernummer in* 10300

☐ Nee > *U kunt geen aanvraag doen*

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

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

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

Straat en huisnummer	Geert Grooteplein 10
Postbus	9101
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	[redacted]
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

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

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

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

2 Over uw aanvraag

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

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum: 01-06-2016 Einddatum: 01-06-2021
3.2	Wat is de titel van het project?	Unraveling the underlying mechanisms of depression
3.3	Wat is de titel van de niet-technische samenvatting?	Onderliggende mechanismen van stress gerelateerde aandoeningen zoals depressie
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC: RU DEC Postadres: Postbus 9101, 6500 HB Nijmegen [redacted] E-mailadres: [redacted]

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

- ☑ Nieuwe aanvraag Projectvergunning € 1.584,00 Lege
- ☐ Wijziging € Lege

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

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

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ■ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☐ Melding Machtiging
- ☒ DEC advies en factuurinformatie

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	[redacted]
Functie	[redacted]
Plaats	Nijmegen
Datum	18-03-2016
Handtekening	[redacted]

2.

Form
Project proposal

- This form should be used to write the project propsal of animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed
- For more information on the project proposal, see our website(www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	Unraveling the underlying mechanisms of depression

2 Categories

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

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

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

3 General description of the project

3.1 Background

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

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

Currently, depression is thought to be caused by an interaction from the environment (stress) and (genetic) predispositions of the individual. Whilst the exact mechanism is not known, it is known that there is a higher risk of developing depression when more stressful life events have occurred (Brown & Harris, 1978, Nemeroff and Vale 2005). Numerous human and animal research show that adverse live events or stress are undoubtedly one of the major risk factors for the development of depression (McEwen 2003, De Kloet et al. 2005, Nemeroff and Vale 2005, Joëls and Baram 2009, Schmidt 2011, Morava and Kozicz 2013). A stressor is something that challenges the individual and is in conflict with the individual's homeostasis. It can be either external or internal, and physical or psychological (Chrousos and Gold 1992, McEwen 2003, De Kloet et al. 2005). In order to maintain homeostasis, the individual must challenge the stressor; one way to do this is by generating the so-called stress response. The best-known and characterized stress response system is the hypothalamic-pituitary-adrenal (HPA)-axis. In this system, upon a challenge, corticotropin-releasing factor (CRF) is released, which via the pituitary gland activates the adrenal glands to release corticosteroids. The released mineralo- and glucocorticoids mediate various important physiological adaptive processes. These adaptive processes involve the recruitment of multiple brain areas and complex brain networks (De Kloet et al. 2005, Pittenger and Duman 2008, Joëls and Baram 2009, Price and Drevets 2012, McEwen et al. 2015). Depending on the stressor, different parts of these networks are recruited that will coordinate different physiological, neuroendocrine, and behavioral aspects of the stress response (Joëls and Baram 2009, Krishnan and Nestler 2010, Morava and Kozicz 2013). If one or more of the stress responsive systems are not functioning properly, the different physiological processes to adequately cope with the stressor cannot be activated properly and the stressor cannot be handled in an adaptive manner increasing the risk to develop depression (McEwen 1998, Brown et al. 2004, De Kloet et al. 2005, Joëls and Baram 2009, Krishnan and Nestler 2010). For example, for over more than two decades it has been known that there is a direct relationship between depression and alterations in the HPA-axis and the CRF system (Nemeroff et al. 1984, Banki et al. 1987, Raadsheer et al. 1994, Bale and Vale 2004, Merali et al. 2004, De Kloet et al. 2005, Pariante and Lightman 2008).

One of the best-known hypotheses for the underlying cause of depression is the monoamine hypothesis. This hypothesis states that a decrease in monoamines, such as serotonin or dopamine, in the synaptic cleft together with environmental factors, such as stress, can be an underlying cause of depression. Most antidepressant medications are based on this theory, and increase the amount of monoamines in the synaptic cleft. Unfortunately, antidepressant treatment alleviates symptoms of depression only after several weeks of medication in only a subset (~50%) of the patient

population (Berton and Nestler, 2006), indicating that there are also other mechanisms involved in the pathology of depression. To develop new therapeutic targets for depression, new concepts and hypothesis are needed described below.

The peptidergic hypothesis

Urocortin 1

One of such new hypothesis that focusses on the mechanisms underlying the stress response adaptation is the peptidergic hypothesis. This hypothesis postulates that if there is an imbalance in peptides involved in initiating and maintaining the stress response, it could lead to increased susceptibility to stress-related disorders. For over two decades, the HPA-axis, involving the neuropeptide CRF, has been considered the main system for controlling the stress adaptation. However, the identification of two CRF receptors (CRF-R1 and CRF-R2) with distinct ligand binding properties, added a new dimension to our view on stress adaptation. Moreover, the discovery of new members of the CRF neuropeptide family, urocortin 1 (Ucn1), urocortin 2 (or stresscopin-related peptide), and urocortin 3 (or stresscopin) has provided important insights into stress adaptation pathways and suggests that stress adaptation involves more systems than the HPA-axis alone (Steckler and Holsboer 1999, Bale and Vale 2004, Joëls and Baram 2009, Vaughan et al., 1995, Hsu and Hsueh, 2001, Lewis et al., 2001, Reyes et al., 2001, Janssen and Kozicz, 2013). Ucn1 is most abundantly expressed in the centrally projecting Edinger-Westphal nucleus (EWcp) (Kozicz et al., 1998; Bittencourt et al., 1999). It has been shown that Ucn 1 is involved in different behaviors such as the suppression of food and water intake (Spina et al. 1996, Jones et al. 1998, Smagin et al. 1998, Coste et al. 2000, Skelton et al. 2000), alcohol drinking (Ryabinin et al. 2012), social behavior (Sajdyk et al. 1999, Skelton et al. 2000) as well as in the stress response, depression, and anxiety (Moreau et al. 1997, Jones et al. 1998, Skelton et al. 2000, Gaszner et al. 2004, Kozicz 2007, Rotzinger et al. 2010, Kormos and Gaszner 2013). Furthermore, in humans it has been found that UCN1 mRNA is up-regulated in brain samples of male, but not female, suicide victims compared to non-depressed controls (Kozicz et al. 2008, Kormos and Gaszner 2013). In addition, these Ucn1 neurons are recruited by various acute stressors and their messenger RNA expression is up-regulated by acute pain and restraint stress (Kozicz et al., 2001; Cunha et al., 2007; Spencer et al., 2012). Our recent data and research by others indicate that an important role in stress adaptation is played by Ucn1 from the EWcp (Gaszner et al., 2004; Korosi et al., 2005; Cunha et al., 2007; Kozicz, 2007).

Orexin

Another family of peptides that seems involved in the stress response are the orexins, also known as the hypocretins, consisting of orexin-A and orexin-B. These peptides are synthesized solely within the lateral hypothalamus and adjacent regions (De Lecea et al., 1998; Sakurai et al., 1998). They bind to two G-protein-coupled receptors, orexin receptor-1 (OXR-1) and orexin receptor-2 (OXR-2) (Sakurai et al., 1998). Several observations suggest that the orexins modulate behavioral state and state-dependent processes. For example, narcolepsy is associated with a decreased concentration of orexin in the cerebrospinal fluid, as well as the number of orexigenic neurons is reduced (Peyron et al., 2000; Thannickal et al., 2000). Furthermore, intracerebroventricular administration of orexin-A or -B increases time spent awake as well as behaviors typical of spontaneous waking and/or stressful, high-arousal conditions, activate the HPA-axis, as well as activates CRF neurons in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala (Kuru et al., 2000; Al-Barazanji et al., 2001; Samson and Taylor 2001; Sakamoto et al., 2004). Orexins are also involved in feeding behavior, the reward system, and the stress response (Sakurai et al., 1998; Kunii et al., 1999). Most of these functions are disturbed in depressed patients, indicating that orexin may be involved in depression. Indeed, several studies found a dysregulation of the orexinergic system in depressed patients (Nollet and Leman 2013, Chen et al. 2015). Also animal research supports the involvement of orexin in depression, different genetic animal models for depression (the Wistar-Kyoto and Flinders Sensitive Line rats) show differences in the orexin system compared to control animals (Allard et al. 2004, Mikrouli et al. 2011, Nollet and Leman 2013, Chen et al. 2015). However, how precisely orexins are involved in the pathology of depression is unknown, as in both human and animal research hypoactivity as well as hyperactivity of the orexin signaling is associated with depression and depressive phenotype (Nollet and Leman 2013, Chen et al. 2015). But with

aforementioned evidence it is reasonable to predict that the orexin system plays a role in depression pathology. In addition to this, various brainstem and basal forebrain regions that are implicated in the regulation of behavioral state of stress, including the locus coeruleus, the medial portion of the preoptic area, the paraventricular hypothalamic nucleus, and the EWcp, contain orexin-containing fibers and orexin receptors (Trivedi et al., 1998; Marcus et al., 2001; Peyron et al., 1998; Cutler et al., 1999; Date et al., 1999; Nambu et al., 1999). However, the exact mechanisms underlying the involvement of these peptides in behavioral and neuropsychological impairments that are observed in depression remain largely elusive.

MicroRNA hypothesis

As described above, dysregulated or altered peptide expression seems to play an important role in the adaptive stress response and depression susceptibility. In recent years a new player as regulator of peptide expression has emerged, namely microRNAs (miRNAs). MiRNAs are small, 21-nucleotide-long units of noncoding RNA which regulate gene expression post-transcriptionally. A miRNA first binds to a miRNA Silencing Complex (miRISC) after which this complex binds to a specific seed region on the 3' untranslated region of an mRNA. As the complex is bound to the mRNA, the mRNA is either degraded, translation is silenced, or in rare cases stimulated. One miRNA can bind up to hundred different mRNAs and, in addition, each mRNA can contain seed regions for hundreds of different miRNAs. To date, miRNAs have been linked to various processes such as metabolism (Ambros et al, 2008), cellular development, and apoptosis (Magni et al, 2014). Furthermore, different miRNAs are also linked to different diseases such as cancer (DeSano & Xu, 2009), Celiac disease (Magni et al, 2014), viral infections (Timoneda et al, 2014), neurodegenerative diseases (Cogswell et al., 2008; Maciotta et al, 2013), as well as psychiatric and stress related disorders (Kocerha et al, 2015). MiRNAs are potential important players in the development of stress related disorders because of their overall abundance in the brain, the potential regulation of different neuropeptides involved in the stress response, or because of their role in the regulation of various metabolic processes. Our preliminary findings on miR-326 show that this miRNA can directly regulate the Ucn1 peptide. Furthermore, others also have shown different miRNAs targeting e.g. CRFR1, glucocorticoid, and corticosteroid signaling (Kocerha et al, 2015, Haramati et al, 2011), all key mediators of the stress response as described above. Studies done on post-mortem brain tissue from depressed patients have shown changed expression levels of several miRNAs which might play a role in depression, including higher levels miR-1202 (Lopez et al, 2014) and lower levels of miR-135 (Issler et al, 2014). An ongoing, comprehensive study done by our lab on the expression patterns of noncoding RNA in the bed nucleus of the stria terminalis (BNST), amygdala, and prefrontal cortex (Broddman's area 25) of patients who suffered from depression and who eventually committed suicide, are showing several other interesting miRNAs including miR-34 and miR-127, which have been linked to stress-related anxiety (Haramati et al, 2011) and cocaine-induced plasticity (Chandrasekar & Deyer, 2011), respectively. As our experiment is ongoing, we will further functionally analyse these potential targets before we select several interesting ones for behavioural testing using the methods described in this project. This study should provide us with a list of microRNAs which are- and which aren't differentially expressed in highly stressed suicidal subjects as compared to a control condition. Whilst we are not sure yet which microRNAs will be tested, we will select those based on: a significant, consistent, differential expression in one or all of the brain areas involved, between the stressed- and non-stressed conditions ($P<0.05$; fold change of at least 1.2; preferentially a similar pattern of expression across brain areas); a similar gene expression of these microRNAs as measured by qPCR in human tissue of these stressed suicidal subjects; possibly similar differential protein expression as measured by luciferase vector assay, should we be able to select a strong regulatory target for the microRNA in question.

Suboptimal mitochondrial function hypothesis

All of the aforementioned processes require a substantial amount of energy mobilization, e.g. the production and regulation of peptides and synaptic plasticity required for a proper stress response. These processes are relative energy expensive processes. If the required amount of energy cannot be mobilized because of genetic defects or because the organism is exhausted, peptide expression and/or synaptic plasticity could be dysregulated. This in turn can lead to an incomplete or failed response to adapt to the stressor, leading to maladaptation. In the brain, most of the energy is

produced by the mitochondria. When mitochondrial function is not adequate for normal daily activities, it causes mitochondrial disorders. Patients with mitochondrial disorder show a 54% lifetime prevalence for depression (Fattal et al. 2007). This is more than two times as high as the incidence in the normal population which is around 20% (Kessler et al., 2003). Also, other studies showed that patients with suboptimal mitochondrial function had a higher incidence for developing depression compared to controls (Suomalainen et al., 1992; Carrozzo et al., 2007; Morava et al., 2006a, b; Koene et al., 2009). This indicates that decreased mitochondrial functioning could be pathological in depression. Furthermore, depressed patients show a decreased mitochondrial functioning in peripheral blood cells (Karabatsiakis et al, 2014). Similar to the monoamine hypothesis, a genetic predisposition in the functioning of the mitochondria together with a stressor may cause the individual to fail to adequately adapt to the stressor and develop stress related disorders such as major depression or anxiety (Morava and Kozicz, 2013). These findings, together with aforementioned required energy mobilization for an adequate stress adaptation, have led to the hypothesis that suboptimal mitochondrial function is involved in the pathology of depression. When not enough energy can be produced because of a decreased mitochondrial functioning, successful adaptation is not achieved and maladaptation may occur that ultimately can lead to stress related disorders such as depression or anxiety. Preliminary findings on mice with decreased mitochondrial functioning show that these animals are more prone to develop depressive behavior when stressed. Also immunohistochemical analysis shows an altered activation of several brain regions involved in the stress adaptation. In order to study this hypothesis a new animal model with decreased mitochondrial function will be used. This animal has a decreased Ndufs4 protein, a subunit of complex I of the mitochondria, because of a gene trap insertion in an early locus of the gene.

As described above, all three hypotheses have an influence on the stress response as well as on depression susceptibility. However, it seems that they also interact with each other. For example, miRNAs have a direct influence on different proteins such as Ucn1 (Aschrafi et al. 2015). Also the different described proteins such as Ucn1 (Lawrence et al. 2004, Townsend et al. 2007, Davidson et al. 2009) and orexin (Sellayah et al. 2011) have a direct influence on the mitochondria. A dysregulation of these proteins could also have a direct influence on mitochondrial functioning. In this way miRNAs can also have an indirect influence on the mitochondria by regulating or dysregulating different proteins. However, it has also been found that different miRNAs have a direct effect on the mitochondria (Chan et al. 2009, Chen et al. 2010). If this is also the other way around, that the mitochondria have a direct effect on the described proteins or miRNAs is unclear, this will also be investigated in this project. It seems that these three hypotheses are tightly linked, however what the mechanisms are, what processes are upstream or downstream, and what their influence is on depression symptoms are, is unknown. This project will contribute to unraveling this.

In this project we will also investigate the fact that depression is not only one disease. Depression has many different symptoms (e.g. Criteria A in DSM V) where only 5 out of 9 symptoms are required for the diagnosis depression. The different symptoms are depressed mood or irritable, decreased interests or pleasure, significant weight change or change in appetite, change in sleep, change in activity, fatigue or loss of energy, guilt/worthlessness, concentration, and suicidality. The different animal models will not only be used because they have interacting mechanisms, but also because they can shed light on potential different mechanisms underlying different symptoms of depression. Because only 5 out of 9 symptoms must be present to diagnose depression, there are many different combinations possible resulting in the fact that not everyone have the same symptoms. This can point towards different mechanisms that we can potentially investigate with the different animal models. For example, this way we can investigate if orexin influences the change in weight, appetite or sleep as depression symptoms, or that the mitochondria are underlying the fatigue, loss of energy or a change of activity. Together with the three different stress models we try to elucidate the potential different underlying causes of depression that can explain the different symptoms with different people.

So, despite several decades of research the current knowledge and therapies for the treatment of depression are not yet sufficient. This is largely because the exact underlying mechanisms of depression are still largely unknown. In this project we will test several new hypothesis for underlying

causes of depression, namely the involvement of the peptides orexin and urocortin as well as microRNAs and suboptimal mitochondrial functioning. This will be done using readily available animal models, namely the orexin KO, Ucn1 KO, and Ndfs4 deficient animals, as well as with mice where a specific miRNA expression is altered. This project also utilizes three different kinds of stressors, namely chronic variable stress, chronic social defeat stress, and acute stress. These three stressors are necessary because it is known that physical and psychological stressor have other effects on different individuals and on underlying mechanisms (Lamb 1979, Briski and Gillen 2001, Kavushansky et al. 2009). The chronic variable stress focusses more on the physical and unpredictable component of the stressor while the social defeat stress focusses more on the psychological component of stress. To determine if the initial stress response is different between WTs and the different animal models in this project an acute stressor is used.

Lastly, an intervention study is utilized to test the influence of different antidepressants on depressive behavior in the stressor that gives the most robust effect in each animal model. This experiment will be done using two different classical antidepressants. These antidepressants will be chosen on for example their effect on the mitochondria. It is known that different antidepressants have a different effect on the mitochondria, some will have a positive effect whilst others have a negative effect on mitochondrial function. This intervention experiment will verify and potentially strengthen results found in first three stress experiments. Classical antidepressants are used because they are readily available and widely used, consequently if this research finds promising new results and uses for these classical antidepressants this could faster lead to more personalized health care.

With this project we aim to shed more light on potential underlying causes and mechanisms for depression. Hopefully with the acquired new insight in the aethiology of depression new and more effective treatments can be generated.

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?

This project aims to provide several important novel insights in the etiology and biological underpinnings of stress related disorders such as depression using various translational animal models. For example, the involvement of mitochondria in the susceptibility in depression, the involvement of certain microRNAs in the pathophysiology of stress related disorders, as well as the involvement of different peptides such as Ucn1, orexin, and CRF in the stress response and depression susceptibility. In addition to this, possible interactions between these different biological underpinnings will also be investigated as well as how much these could contribute to the pathology of various Category A depression symptoms (DSM V) like fatigue, sleep problems, psychomotor retardation, etc.. The use of novel hypotheses, as outlined in section 3.1, in combination with well-validated models for stress-related psychological diseases with the focus on depression, will help us better understand the underlying mechanisms of stress related psychiatric diseases as well as a better understanding of the various category A symptoms of depression. Consequently, it could also give new insights in identifying novel therapeutic targets and treatment strategies of major depression. In addition, important data will be acquired to better understand fundamental mechanisms contributing to stress adaptation, and consequently will increase our insight into neuronal processes that may underlie unsuccessful adaptation to stress and depression susceptibility. Ultimately, this may lead to the development of new treatment targets or the re-categorization of current treatments which is investigated in experiment 4. In order to investigate the interaction between the above detailed mechanisms in depression vulnerability, the various mechanisms mentioned in the background are

examined in parallel. We will be able to identify different behavioral, physiological, and endocrine aspects of depression that can be correlated/compared to similar parameters in humans giving this project a great translational value.

Similar experiments investigating stress-related disorders were already performed in the past in our lab including experiments with Ndufs4def, orexin KO, and Ucn1 KO mice, giving us experience with these types of experiments. This allows us to work efficiently and with the least amount of distress for the animals. Also, because of this experience and the available knowledge, the main objectives should be achievable and realistic within the duration of the project. This project will be a continuation of the research that has already been performed in this lab towards unraveling the underlying mechanisms of depression. Previous experiments encompass miRNA 326 and Ucn1 (Aschrafi et al. 2015), orexin and stress (Emmerzaal et al. 2013), Ucn1 and depression (Kozicz 2007, Spencer et al. 2012), as well as mitochondrial function and depression (Emmerzaal et al. 2015).

3.3 Relevance

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

It has been estimated that one in five individuals will develop depression in some point in life and in 2010 there were 298 million separate cases of depression recorded globally, with an average duration of 37.7 weeks (Ferrari et al, 2013). At that time depression was the second leading cause of burden of disease worldwide (Ferrari et al, 2013), whilst the World Health Organization (WHO) estimated that depression would be the leading cause of disease by the year 2020. At this moment the WHO reports that depression is the leading cause for disease with worldwide 350 milion people suffering from depression of all ages. Depression not only has a profound effect on the individual but also on his/her family and society as a whole. Despite decades of research towards the pathogenic mechanisms behind depression, the neurobiology underlying this complex disorder remains largely elusive. A consequence is that treatment options at the moment remain poor, with up to 40% of patients not responding to current treatment methods (Fava, et al, 1996; Berton and Nestler, 2006; Saad Al-Harbi, 2012). Therefore, understanding the (neuronal) mechanisms underlying fundamental biological processes in adaptation to stressors are of great importance (as the link between stress adaptation and depression indicated in section 3.1). Despite several decades of research that have identified several possible underlying pathologies, progress in understanding depression, and related disorders overall, has been slow and the search for new therapeutic targets and approaches is necessary. In this context, fundamental animal research utilizing well-validated animal models of depression is therefore of great importance to reveal novel mechanisms of stress-related psychiatric diseases that can lead to novel treatment strategies.

3.4 Research Strategy

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

The main objective of this project is to investigate underlying mechanisms that mediate the behavioral, endocrine and physiological alterations seen in depression, a stress-related disorder. In these experiments the different genes of interest and possible underlying mechanisms described in section 3.1 will be investigated using different animal models. These animals are bred for a specific genetic knockout/knockdown and therefore have a general change in expression of the gene of interest. In these experiments we will be using mice which lack orexin and Ucn1. We will also use an Ndufs4 deficient mouse model to induce a decreased mitochondrial function in these animals. These animals are readily available in our lab. Furthermore, also two different miRNAs will be tested through either lenti-/adeno-viral injections or through specific ‘floxing’ of animals and

employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The specific miRNA will be chosen after a comprehensive investigation in suicide patients that is ongoing at this moment as described in section 3.1. Using these genetically modified animals, we will aim to investigate whether changes in the expression of these genes or miRNAs, coupled with different methods of stress induction influence depression susceptibility. To investigate depression susceptibility three different stress paradigms will be used, 1) a chronic variable stress paradigm, 2) chronic social defeat stress paradigm, and 3) an acute stress paradigm. 4) After these three experiments an intervention study with different antidepressants will be utilized with the stress model that gives the most robust findings in the animals.

The five different animal models (Ucn1 KO, orexin KO, Ndufs4def, altered miRNA mice) will be used in four different experiments. All these animal models will be used to study the following:
1) Investigate the effect of unpredictable chronic stress on depression susceptibility.
For this experiment the different animal models are subjected to a chronic variable stress paradigm and their depression susceptibility is determined via various behavioral tests and biochemical parameters.
The questions to be answered in this experiment are:
- Is depression susceptibility after a chronic variable stress paradigm influenced by altered peptide levels, mitochondrial function, or miRNA abundance?
- What are the specific effects of these altered parameters on the animals behavior after the stressor?
- What biochemical parameters underlie and influence the potential depression susceptibility?

2) Investigate the effect of social defeat stress on depression susceptibility.
For this experiment the different animal models are subjected to social defeat stress. This will be induced via a resident intruder paradigm. Depression susceptibility is again determined via various behavioral tests and biochemical parameters.
The questions to be answered in this experiment are:
- Is depression susceptibility influenced by altered peptide levels, mitochondrial function, or miRNA abundance after social stress?
- Are other mechanisms involved in depression susceptibility after social stress as compared to chronic variable stress?

3) Investigating differences during the acute stress response as possible underlying causes for depression susceptibility.
In this experiment the different animal models will be challenged by an acute stressor to determine if the initial stress response is already altered in these animals.
This experiment addresses the following questions:
- Can the potential differences found during the chronic stressors be explained by a difference during the acute stress response?
- Are different acute stress parameters negatively altered in the different animal models?
- Is there a difference in neuronal network activation during the initial stress response?

4) The effect of different antidepressants on reducing depression susceptibility
In this experiment one of the three stressors will be used in an intervention experiment. The animals will be stressed while different groups of animals will receive different treatments. These different treatments are for example based on the effect at the mitochondria.
With this experiment we can address the following questions:
- Can the enhancement or decrease of mitochondrial function by antidepressants influence depression susceptibility?
- Are current antidepressants effective in the treatment of depressive behavior in these different animal models?

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.

First we plan to investigate the effects of different stressors on depression susceptibility in the different animal models as explained in the previous sections. We will use mice which lack the peptides Ucn1 and Orexin, as well as a new mouse model with decreased mitochondrial function because of a lower Ndufs4 expression. These animals are readily available and present in our lab. To investigate the influence of miRNA abundance we will use specifically engineered viral vectors that can be injected locally, to induce changes in microRNA abundance. To investigate depression susceptibility of these genetically modified animals they will be subjected to three different well validated stress paradigms; 1) chronic variable stress (Pittenger and Duman 2008, Hill et al. 2012, Franceschelli et al. 2014), 2) chronic social defeat stress through a resident intruder paradigm (Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991, Rygula et al. 2005), and 3) acute stress (Katz et al. 1981, Fullerton et al. 2004, Bogdan and Pizzagalli 2006).

1) The chronic variable stress model is based on the learned helplessness aspect of stress. The animal is not able to control or predict the stress it will face, due to its unpredictable– and chronic nature. The chronic stress will consist of a variable stress paradigm where for 21 consecutive days a stressor and/or a behavioral test will be presented to the animals. These stressors will be presented in a randomized order where each stressor is presented an equal number of times. The duration of the stressor can be between one hour and an overnight time period. All animals in the stressed group will receive the same stressors.

2) The social defeat stress model is based on stress originating from the experience of aggression and submission. This will be done via a social defeat paradigm. Previous research has shown social defeat stress to be a viable animal model for depression, that induces a depressive phenotype (Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991; Rygula et al., 2005) and an enduring activation of the HPA axis (Covington & Miczek, 2005; Koolhaas, De Boer, De Rutter, Meerlo, & Sgoifo, 1997).

3) Acute stress will be used to study changes in the initial phase of the stress response that could eventually lead to a depressive phenotype. The stressor can be for example a series of electric foot shocks, restraint, lipopolysaccharide (LPS) injection, or forced swimming.

After each of the aforementioned stress procedures several well validated behavioral tests will be performed in order to investigate normal- and depression-like behavior. Behavioral tests that will be used are for example the Porsolt swim test, sucrose preference test, novelty suppressed feeding, open field test, Rotarod, and grip test. In addition to these tests, the animals will be implanted with a telemetry sensor that can measure autonomous functions such heart rate and core body temperature. This gives information about the rhythm and possible autonomous disturbances the animals may have or develop during the stress paradigm. In order to measure brain structure and function in vivo, neuroimaging techniques are used, such as Diffusion Tensor Imaging (DTI), resting state functional MRI (rs-fMRI) and Magnetic Resonance Spectroscopy (MRS). Shortly after the last stressor- and behavior experiments the animals will be sacrificed and biological material will be collected for further histological and biochemical analyses.

4) Lastly, after these three experiments, an intervention study will be done utilising the stress model that gives the most robust findings in the animals. This can differ for the different genetically modified animals. The intervention will encompass two different classical antidepressants. Different antidepressants will be used because it is for example known that some antidepressants have a positive while others have a negative effect on the mitochondria, possibly influencing depression susceptibility for better or for worse (Klinedinst and Regenold 2015). The choice of the specific

antidepressants will be made after all three stress experiments are executed. At this moment this choice cannot be made because it depends on the obtained results as well as the advances that are being made for different effects, and potentially newly discovered antidepressants.

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 individual components of this project focus on understanding the influence of the different described peptides, mitochondrial function, and miRNAs on depression susceptibility after stress and the potential interactions between these different mechanisms.

The four proposed animal procedures will be executed in succession with each other (see figure 1). This flowchart will be used for each of the five proposed animal models (orexin KO, Ucn1 KO, Ndufs4 deficient, and two virally injected groups). Between the different animal procedures there will not be a go/no-go evaluation moment.

Each animal procedure is first executed with behavioral testing and biochemical and histological analysis only. After the experiment is completed, there is a go/no-go evaluation moment, if differences are found between WT and genetically modified animals MRI experiments will also be utilized (Figure 1). The same paradigm with behavioral tests is executed before the MRI. If no differences are found no MRI experiment will be implemented (see figure 1) and the next animal procedure can be executed. The MRI protocol includes DTI, rs-fMRI, and MRS to investigate functional and structural differences between animals in vivo.

Before the start of animal procedure 4, there is another go/no-go evaluation moment. All three animal procedures are evaluated and compared for that specific animal model. The paradigm that gave the most reliable and robust results will be used for the intervention experiment to minimize the number of animals used.

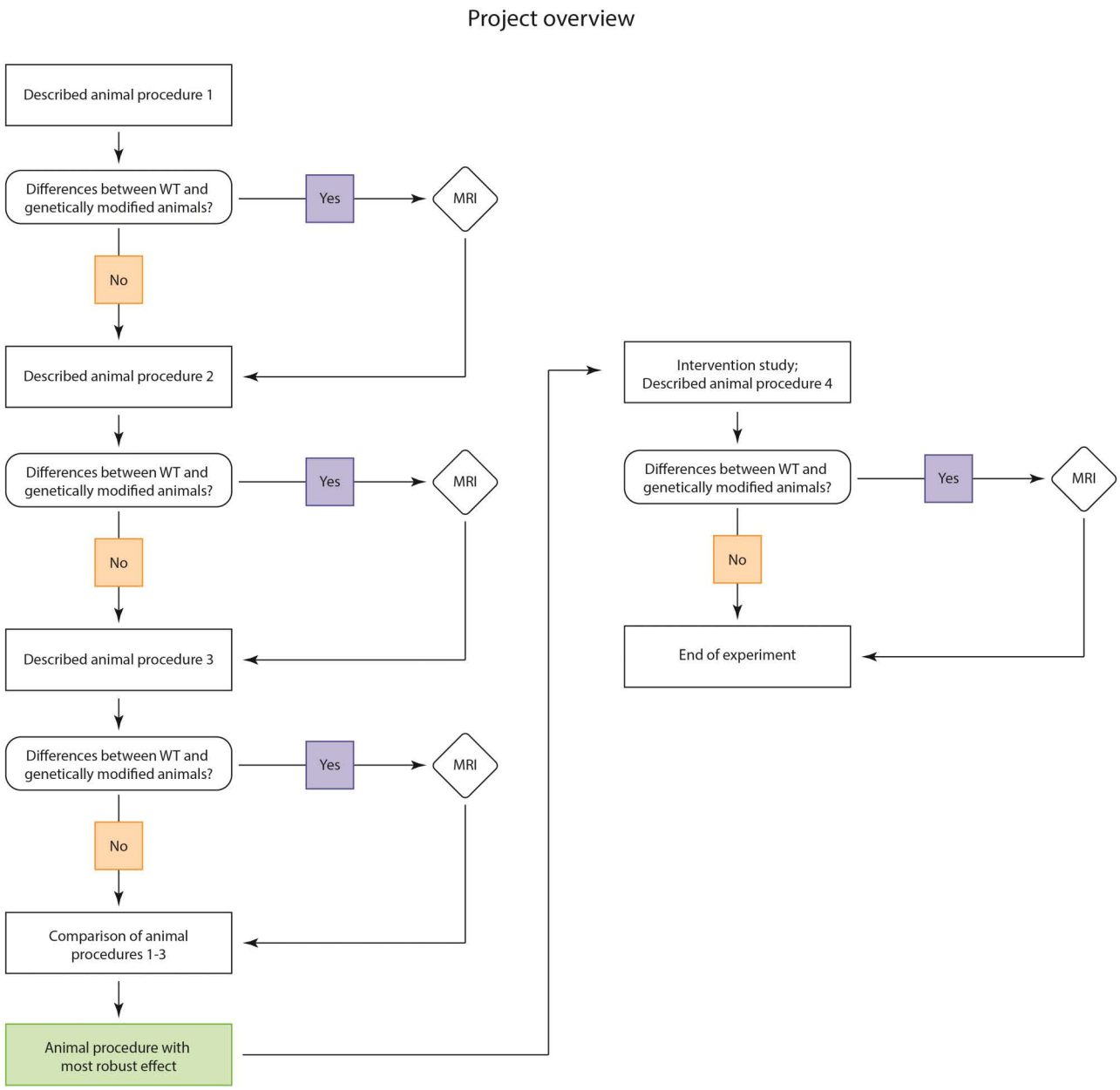

Figure 1: Project overview

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	Chronic variable stress paradigm
2	Chronic Social defeat stress
3	Acute stress paradigm
4	Intervention experiment

3.

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 1	Type of animal procedure Chronic variable stress paradigm

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.

Different animal models will be used in order to study whether altered protein expression, and/or decreased mitochondrial function can affect depression susceptibility. The protein expression is either constitutively altered (orexin KO and Ucn1 KO mice) or can be influenced with miRNAs. Orexin KO and Ucn1 KO mice are readily available and used in this project because these proteins have been shown to be involved in depression susceptibility as described in section 3.1 of the project proposal and also to continue previous research in our lab. Urocortins 2 and 3 will not be used in this project because although they are paralogous to each other and are part of the CRF family, they are distinct from CRF and urocortin 1 (Rotzinger et al. 2010). The specific miRNAs of interest will be determined after the comprehensive study mentioned in section 3.1 of the project proposal is finished, as of now miR-34 and miR-127 seem promising targets. When the miRNAs of interest are determined they will be influenced either through lenti-/adeno-viral injections or through specific 'floxing' of animals and employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The actual choice will depend on the gene/microRNA in question, and whether there is a 'floxed' animal model available. To investigate the influence of decreased mitochondrial functioning on depression susceptibility a new mouse model with a gene trap insertion in the Ndufs4 allele will be used. This genetic alteration results in a premature termination of the coding sequence and deficiency of the NDUFS4 protein. This deficiency results in a decreased mitochondrial complex I and III activity of the electron transport chain, but the animals behavior remains normal under basal conditions.

The different above described animal models will be subjected to a chronic variable stress paradigm, a well validated and often used paradigm to induce experimental depressive-behavior in animals. We will assign both the genetically modified animals as well as WT mice randomly to the chronic stress or control group. Control mice will be exposed to similar conditions compared to mice in the chronic variable stress group, but without exposing them to the stressor. During and after the chronic stress paradigm mice will be subjected to several behavioral tests to monitor the effect of the chronic stress on the animal's behavior. Different behavioral tests will measure depressive behavior (e.g. novelty suppressed feeding, forced swim test, tail suspension test) as well as well as general behavior such as locomotor activity (e.g. RotaRod, Phenotyper, open field). The weight of the animal will also be measured several times during the paradigm. Shortly after the last stressor or behavioral test the animals will be sacrificed, blood and biological material will be collected, such as the brain, and several other organs. If robust changes are found, these studies are followed up by magnetic resonance imaging (MRI) to study brain structure and function, including DTI, rs-fMRI, and MRS. The neuroimaging will be executed on a different group of animals because it is known that isoflurane, that is used to anesthetize the animals, has an influence on mitochondrial function and on the stress response parameters. These animals will also undergo the different behavioral tests.

In order to determine the effect of the stressor on autonomic functions in the different animal models such as body temperature, as well as overall activity and sleep patterns, a telemetry sensor that can measure these will be implemented in the mice. This will both be in WT as well as genetically modified animals. The sensor will be implemented in these animals minimal two weeks before the stress procedure in order for the animals to recover from surgery. In this time period it will also be possible to set a baseline for each animals before the stress experiment.

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or mRNA in the brain, and mitochondrial function. These primary readout parameters will be used because it is known for example body weight decreases, plasma corticosterone increases, and mitochondrial function is altered during the stress paradigm. Furthermore these read out parameters are well-validated markers for depressive behavior, widely used, and easily measured. If a robust statistically significant difference between the different groups is found, neuroimaging will also be conducted. In order to correlate biochemical, behavioral and neuroimaging data to each other the animals that undergo neuroimaging will also be behaviorally tested.

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

Our aim is to compare genetically modified- or virally injected animals to WT littermates (non-genetically modified or control virally injected (injected with a virus containing a scrambled target sequence) respectively), by exposing them to a chronic stress paradigm. During and after the stress paradigm the animals will be exposed to several standard behavioral tests devised to measure depression-related behavior. Mice will randomly be assigned to the either the control group or the stress group.

The telemetry sensor that will be implanted can measure core body temperature and activity. Animals will be operated minimally two weeks prior to the stress induction to allow enough time for the animals to recover. The telemetry sensor will be placed subcutaneaus under general anesthesia (1.5 – 2% isoflurane inhalation).

The miRNA abundance will be influenced via local viral injections in the brain. The virus will be injected during the same operation as the telemetry sensor implantation to allow enough time for incubation and also recovery from surgery and to reduce discomfort of two separate surgeries. The viral injection is done while the animal is placed in a restraint / stereotactic apparatus, whilst the viral construct can be delivered through a thin syringe and needle.

The genetically modified animals, as well as the WT littermates, are randomly assigned to either the experimental- or the control condition. Both groups will follow similar protocols, with the only difference that the experimental group will be stressed, and the control group will not receive stress. In this experiment, chronic stress is induced by a chronic variable stress paradigm. This paradigm consists of 21 successive days where each day a stressor and/or a behavioral test is presented to the animal. Each animal in the stress group receives the same stressors and these stressors will be presented in a randomized order where each stressor, mentioned in table 1, is presented an equal number of times (except for the behavioral tests mentioned in the table). The duration of the stressor varies between one hour and an overnight time period, depending on the stressor. The stressors that will be used are for example restraint stress, cold stress, confinement, continuous light overnight, social isolation overnight, soiled cage overnight, shaking stress, cage tilting, overnight food deprivation (for more details see table 1). This paradigm is based upon previously experiments as well as described regimens and is well validated and a proven mouse model for depressive behavior in rodents (Stout et al. 2000, Willner 2005, Deussing 2007, Franceschelli et al. 2014).

Table 1. Different stressors used during the chronic variable stress paradigm. These stressors will be presented to the animals in a randomized order one a day.

Stressor	Average duration	Short description	Discomfort level
Confinement	Repeated 1h periods	Animals are placed in a small cage	Moderate
Cold stress	1 hour	Animals are exposed to 4°C	Severe
Continuous light	Overnight	Animals are exposed to continuous light overnight	Moderate
Social isolation	Overnight	Animals are isolated overnight; one mouse/cage. This can be for example combined with an Phenotyper for behavioral analysis	Mild
Soiled cage	Overnight	Bedding material will be soiled with water (±250 ml)	Severe
Shaking stress	1 hour	Cages are placed on an orbital shaker (±100 rpm)	Moderate
Restraint	30 min	Animals are placed in a plastic restrainer	Moderate
Cage tilting	Overnight	The home cage of the animals will be tilted overnight	Moderate
Food deprivation	Overnight	Animals will not have access to ad libitum food overnight	Moderate
Novelty suppressed feeding	10 min	Animals will be placed in a novel environment where a food pallet is placed in the middle; executed after overnight deprivation	Mild
Porsolt swim test	10 min	Animals are placed in a cylinder filled with water and are forced to swim for 6 minutes	Severe
Tail suspension	10-15 min	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces.	Moderate

At the end of the stress paradigm general and depressive related behavior of the animals will be analyzed with several different behavioral experiments. For example the Porsolt swim test, tail suspension test, sucrose preference test, novelty suppressed feeing, open field test, Rotarod, splash test, and grip test. The behavioral tests that have a higher discomfort for the animals, Porsolt swim test and tail suspension test, will not be executed on the same animal. These two tests will be executed with two other milder behavioral tests. Short descriptions of these tests are included in table 2. Each behavioral test is executed once at the end of the stress paradigm.

Table 2. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

On the 21st day, shortly after the lasts behavioral test, the animals will be sacrificed and blood will be taken. The sacrificing method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood, and several other organs will also be collected for additional analyses.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size was based and calculated using standard deviations from the behavioral test with the most variation (FST) from previous experiments. The effect size was $d=0.982$, giving a minimal number of animals of 14 per group. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 14 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly besides the stress paradigm. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the experimental- or control condition. This will be to strengthen the study design by limiting variation between groups due to experimenter buis and housing influences.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the new Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

Previous studies and experience in our lab using comparable chronic variable stress-paradigms have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. We need a minimum of 14 animals per group, however, we do not want to expose each animal to more than 3 behavioral tests post-stressor/non-stressor and we aim to do 4 to 6 behavioral tests. Therefore another group of animals has to be added for the additional behavioral tests, totaling at 28 animals per group (see table 3). The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of 14 animals per group, for details see table 3.

Table 3. Maximal number of animals necessary

Animal model	Number of animals per group				Total
	No-MRI		MRI		
	Control	Stress	Control	Stress	
Orexin KO / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
Ucn1 KO / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
Ndufs4def / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
MiRNA 1 / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
MiRNA 2 / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
Total	140 / 140	140 / 140	70 / 70	70 / 70	900

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	900	Adult

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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The model we chose is one of the best validated animal models for stress-related psychopathology, and although it uses a chronic stress exposure, it results in relative mild/moderate physical and psychological distress/harm compared some other stress-paradigms. The behavioral tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioral changes. Viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anesthesia (via 1.5% isoflurane inhalation) in order to minimize animal suffering. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that chronic stress causes discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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.

Non-applicable

Accommodation and care

F. Accommodation and care

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

[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?

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

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

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

During viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of 1.5% isoflurane inhalation. Otherwise no anesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

During the chronic stress paradigm we will not apply anesthesia or analgesia. When mitochondrial function will not be tested, and when perfusion will be used as a means of sacrifice, animals are first anesthetized by means of 1.5% isoflurane inhalation.

I. Other aspects compromising the welfare of the animals

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

Stress will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, a chronic stressor has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to chronic stress, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful will be implemented in the chronic stress paradigm to monitor their behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The chronic variable stress paradigm is a widely used approach to investigate stress adaptation and stress associated disorders such as depressive behavior. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behavior (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the chronic stress paradigm or have complications from surgery. Animals will constantly be monitored for signs of sickness, infection, excessive weight loss, or other signs of diminished well being. If an animal during the

experiment shows a body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or veterinarian. Because of the non-invasive nature of the stressors and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The stressor is of a non-invasive nature whereby the incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments. Based upon our previous experience with similar experiments with the Ucn1 KO mice, we expect to have less than 3% of the animals develop these criteria.

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 the animals experience at the end of the chronic stress paradigm and behavioral tests is considered severe. The effect of the behavioral tests is expected to be mild to severe, depending on the behavioral test. The cumulative discomfort of the animals subjected to chronic stress is severe; for control mice this cumulative discomfort will moderate or severe, as is classified is Annex VIII of the Directive 2010/63/EU. The discomfort severity of the control mice depends on the cluster of behavioral tests, if the cluster includes the Porsolt swim test it will be severe (50% of the control animals), if not and it includes the tail suspension test it will be moderate (50% of the animals). In total, 75% of the animals will experience severe discomfort and 25% of the animals will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without

these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 2	Type of animal procedure Chronic Social defeat stress

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.

Different animal models will be used in order to study whether altered protein expression, and/or decreased mitochondrial function can affect depression susceptibility. The protein expression is either constitutively altered (orexin KO and Ucn1 KO mice) or can be influenced with miRNAs. Orexin KO and Ucn1 KO mice are readily available and used in this project because these proteins have been shown to be involved in depression susceptibility as described in section 3.1 of the project proposal and also to continue previous research in our lab. Urocortins 2 and 3 will not be used in this project because although they are paralogous to each other and are part of the CRF family, they are distinct from CRF and urocortin 1 (Rotzinger et al. 2010). The specific miRNAs of interest will be determined after the comprehensive study mentioned in section 3.1 of the project proposal is finished, as of now miR-34 and miR-127 seem promising targets. When the miRNAs of interest are determined they will be influenced either through lenti-/adeno-viral injections or through specific 'floxing' of animals and employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The actual choice will depend on the gene/microRNA in question, and whether there is a 'floxed' animal model available. To investigate the influence of decreased mitochondrial functioning on depression susceptibility a new mouse model with a gene trap insertion in the Ndufs4 allele will be used. This genetic alteration results in a premature termination of the coding sequence and deficiency of the NDUFS4 protein. This deficiency results in a decreased mitochondrial complex I and III activity of the electron transport chain, but the animals behavior remains normal under basal conditions.

The different animal models will be subjected to a chronic social stressor by using the well-validated Resident-Intruder paradigm, which uses repeated aggressive confrontations to induce social defeat. The animals will be subjected to a 10-day period of daily confrontations, followed by a social dominance test. Previous research has shown social defeat stress to be a good animal model for depression, whereby not only depressive behavior is induced (Kudryavtseva, et al., 1991; Rygula et al., 2005), but also a robust enduring activation of the HPA axis (Covington & Miczek, 2005; J M Koolhaas er al., 1997). We will assign both the genetically modified animals as well as WT mice randomly to the social defeat stress or control group. Control mice will be exposed to similar conditions compared to mice in the chronic stress groups, but these mice will be introduced to a non-aggressive mouse strain. To prevent the stress of confrontation, the cage will be fitted with a barrier to allow the animals to smell, see, and hear each other, however which will prevent the actual interaction. After the stress paradigm mice will be subjected to several behavioral tests to monitor the effect of the stress on the animal's behavior.

If robust changes are found, these studies are followed up by magnetic resonance imaging (MRI) to study brain structure and function, including DTI, rs-fMRI, and MRS. The neuroimaging will be executed on a different group of animals because it is known that isoflurane, that is used to anesthetize the animals, has an influence on mitochondrial function and on the stress response parameters. These animals will also undergo the different behavioral tests.

Different behavioral tests will focus on depressive behavior in these animals. Also general behavior such as locomotor activity will be tested (e.g. RotaRod, Phenotyper, and Open Field). The weight of the animal will also be measured several times during the paradigm. Shortly after the last behavioral test the animals will be sacrificed, blood and biological material will be collected, such as the brain, and several other organs. In order to determine the effect of the stressor on autonomic functions in the different animal models such as body temperature, as well as overall activity and sleep patterns, a telemetry sensor that can measure these will be implemented. This will both be WT as well as genetically modified animals. The sensor will be implemented in these animals minimal two weeks before the stress procedure in order for the animals to recover from surgery. In this time period it will also be possible to set a baseline for each animals before the stress experiment.

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or mRNA in the brain, and mitochondrial function. These primary readout parameters will be used because it is known for example body weight decreases, plasma corticosterone increases, and mitochondrial function is altered during the stress paradigm. Furthermore these read out parameters are well-validated markers for depressive behavior, widely used, and easily measured. If a robust statistically significant difference between the different groups is found, neuroimaging will also be conducted. In order to correlate biochemical, behavioral and neuroimaging data to each other the animals that undergo neuroimaging will also be behaviorally tested.

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

Our aim is to compare genetically modified- or virally injected animals to WT littermates (non-genetically modified or control virally injected (injected with a virus containing a scrambled target sequence) respectively), by exposing them to a chronic social defeat paradigm followed by several standard behavioral tests devised to measure depression-related behavior. Mice will randomly be assigned to the either the control group or the stress group.

The telemetry sensor that will be implanted can measure core body temperature and activity of the mice. Animals will be operated minimally two weeks prior to the stress induction to allow enough time for the animals to recover. The telemetry sensor will be placed subcutaneous under general anesthesia (1.5 – 2% isoflurane inhalation).

The miRNA abundance will be influenced via local viral injections in the brain. The virus will be injected during the same operation as the telemetry sensor implantation to allow enough time for incubation and also recovery from surgery and to reduce discomfort of two separate surgeries. The viral injection is done while the animal is placed in a restraint / stereotactic apparatus, whilst the viral construct can be delivered through a thin syringe and needle.

The genetically modified animals, as well as the healthy WT littermates, are randomly assigned to either the experimental- or the control condition. Both groups will follow similar protocols, with the only difference being that the experimental group will be introduced to very aggressive BALB/cJ mice while the control animals will be introduced to C57BL/6J mice. The control mice will be in the same cage as the resident and while the mice are being able to see, hear, and smell each other they are not able to have physical contact. Over a ten day period the animals will be introduced daily to

a different resident. Our particular confrontation protocol is similar to DEC-nr.: 2013-235, and the protocol by Koolhaas et al (2013). Animals will be in each others' presence for no longer than 10 minutes (attack latency), in which the attacks can take place. An session will last no longer than 5 minutes after the first attack.

At the end of the stress paradigm general and depressive related behavior of the animals will be analyzed with several different behavioral experiments. For example the Porsolt swim test, tail suspension test, sucrose preference test, novelty suppressed feeing, open field test, Rotarod, splash test, and grip test. The behavioral tests that have a higher discomfort for the animals, Porsolt swim test and tail suspension test, will not be executed on the same animal. These two tests will be executed with two other milder behavioral tests. Short descriptions of these tests are included in table 4. Each behavioral test is executed once at the end of the stress paradigm.

Table 4. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

Afterwards blood samples will be acquired and the animals will be sacrificed whereby the method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood plasma, and several other organs will also be collected for additional analysis.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size was based and calculated using standard deviations from the test with the most variation from previous experiments. The effect size was $d=1.05$, giving a minimal number of animals of 12 per group. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 12 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly besides the social stress paradigm. During the social stress paradigm residents/intruders will be mixed constantly in a way that residents and intruders will never be confronted twice with each other to avoid recognition. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the experimental- or control condition. These considerations will be to strengthen the study design by limiting variation between groups due to experimenter bias and housing influences.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

To induce chronic stress through a social defeat paradigm two main groups of animals are needed: a group of residents and a group of intruders (the animals of interest, including the below described genetically modified animals). The residents are required to be more aggressive and dominant that the intruders, in order to induce a stable level of stress and social defeat. BALB/cJ mice will be used as residents, besides being slightly bigger compared to the C57BL/6J mice, they are also more aggressive (Fairless et al., 2012, Velez, et al., 2010). Besides the aggressive BALB/cJ mice, we will use WT littermates as non-aggressive normal behaving animals as a control condition.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

Previous studies and experience in our lab using comparable chronic social defeat stress-paradigms have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. We need a minimum of 12 animals per group, however, we do not want to expose each animal to more than 3 behavioral tests post-stressor/non-stressor and we aim to do 4 to 6 behavioral tests. Therefore another group of animals has to be added for the additional behavioral tests, totaling at 24 animals per group (see table 3). The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per

animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

The number of residents required for this experiment is the same as the number of intruders described above (see table 5). This ensures that each intrudes see a different resident every time to avoid recognition between the animals.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of 12 animals per group, for details see table 5.

Table 5. Maximal number of animals necessary

Animal model	Number of animals per group				Total
	No-MRI		MRI		
	Control	Stress	Control	Stress	
Orexin KO / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
Ucn1 KO / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
Ndufs4def / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
MiRNA 1 / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
MiRNA 2 / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
Total	120 / 120	120 / 120	60 / 60	60 / 60	720

	WT	BALB/cJ	WT	BALB/cJ	
Residents	240	240	120	120	720
Total:	480	480	240	240	1440

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	1440	Adult

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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The model we chose is one of the best validated animal models for stress-related

psychopathology, and although it uses a chronic stress exposure, it results in relative mild/moderate physical and psychological distress/harm compared some other stress-paradigms. The behavioral tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioral changes. Viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anesthesia (via 1.5% isoflurane inhalation) in order to minimize animal suffering. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that chronic social defeat stress causes discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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.

Non applicable

Accommodation and care

F. 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?

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

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

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

During viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of 1.5% isoflurane inhalation. Otherwise no anesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

During the social defeat paradigm we will not preform anesthesia or analgesia. When mitochondrial function will not be tested, and when perfusion will be used as a means of sacrifice, animals are first anesthetized by means of 1.5% isoflurane inhalation.

I. Other aspects compromising the welfare of the animals

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

Stress and single housing of the residents will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, a chronic stressor has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. . During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anaesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to social defeat stress, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful are executed after the resident intruder paradigm to determine their behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The stressors' nature is non-invasive, and duration will be strictly monitored, in order to minimize physical- and mental distress, whilst still being able to induce differences between treatment groups. We have selected a maximum time of each separate confrontation (10 minutes), with a maximum of 5 minutes after the first attack. Also, we will make sure that no excessive physical harm is done; we will separate animals and terminate the confrontation prematurely if we deem the risk of excessive physical harm too high. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behaviour (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the chronic stressor or have complications from surgery. Animals will constantly be monitored for signs of sickness, infection, large open wounds, other extreme physical harm, excessive weight loss, or other signs of diminished wellbeing. If an animal during the experiment shows a body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or veternarian. Because of the non-invasive nature of the stressor and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The stressor is of a non-invasive nature, whereby we will closely monitor the attacks, perform proper wound care, and make sure the animals eat well. The incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments. We expect to have less than 5% of the animals develop these criteria.

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 the animals experience from the intruder’s point of view in the resident intruder paradigm is considered moderate to severe, depending on the resident they will face. The effect of the behavioral tests is expected to be mild or severe, depending on the behavioral test. The cumulative discomfort of the animals subjected to the aggressive Balb/cJ mice (50%) in combination with the behavioral tests is severe; for the intruders which will face the C57BL/6J mice this cumulative discomfort will be moderate to severe depending on the behavioral tests used. The discomfort for the residents will be moderate, as is classified is Annex VIII of the Directive 2010/63/EU.
The mice that will face the C57BL/6J mice and receive the Porsolt swim test will have severe discomfort (50%) while the animals that face the C57BL/6J mice and do not receive the Porsolt swim test will have moderate discomfort (50%). In total, 37.5% of the animals (including both residents and intruders) will experience severe discomfort and 62.5% of the animals will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 3	Type of animal procedure Acute stress paradigm

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.

Different animal models will be used in order to study whether altered protein expression, and/or decreased mitochondrial function can affect depression susceptibility. The protein expression is either constitutively altered (orexin KO and Ucn1 KO mice) or can be influenced with miRNAs. Orexin KO and Ucn1 KO mice are readily available and used in this project because these proteins have been shown to be involved in depression susceptibility as described in section 3.1 of the project proposal and also to continue previous research in our lab. Urocortins 2 and 3 will not be used in this project because although they are paralogous to each other and are part of the CRF family, they are distinct from CRF and urocortin 1 (Rotzinger et al. 2010). The specific miRNAs of interest will be determined after the comprehensive study mentioned in section 3.1 of the project proposal is finished, as of now miR-34 and miR-127 seem promising targets. When the miRNAs of interest are determined they will be influenced either through lenti-/adeno-viral injections or through specific 'floxing' of animals and employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The actual choice will depend on the gene/microRNA in question, and whether there is a 'floxed' animal model available. To investigate the influence of decreased mitochondrial functioning on depression susceptibility a new mouse model with a gene trap insertion in the Ndufs4 allele will be used. This genetic alteration results in a premature termination of the coding sequence and deficiency of the NDUFS4 protein. This deficiency results in a decreased mitochondrial complex I and III activity of the electron transport chain, but the animals behavior remains normal under basal conditions.

The different animal models will be subjected to an acute stressor to investigate the immediate susceptibility to depression. Acute stress will be induced by using one of a variety of well-validated methods, all animals in the same experiment will receive one and the same stressor. We will assign both these genetically modified animals as well as WT mice randomly to the acute stress or control group. Control mice will be exposed to similar conditions compared to mice in the acute stress group, but without exposing them to the stressor. On these different animal models several behavioral tests will be executed after the stressor. After the last behavioral test, the animals are sacrificed and several biochemical and histological analyses will be performed. If robust changes are found, these studies are followed up by magnetic resonance imaging (MRI) to study brain structure and function, including DTI, rs-fMRI, and MRS. The neuroimaging will be executed on a different group of animals because it is known that isoflurane, that is used to anesthetize the animals, has an influence on mitochondrial function and on the stress response parameters. These animals will also undergo the different behavioral tests.

In order to determine the effect of the stressor on autonomic functions in the different animal models such as body temperature, as well as overall activity and sleep patterns, a telemetry sensor that can measure these will be implemented. This will both be WT as well as genetically modified animals. The sensor will be implemented in these animals minimal two weeks before the stress procedure in order for the animals to recover from surgery. In this time period it will also be possible to set a baseline for each animals before the stress experiment.

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or

Table 6. Short descriptions of acute stressors that can be used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Electrical foot shocks	The animals are placed in a skinner box where they will receive a series of inescapable unpredictable foot shocks with a duration of 20-30 min.	Severe
Physical restraint	The animals are placed in a plastic restrainer for 30 min to 1 h	Moderate
Tail suspension	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes.	Moderate
LPS injection	The animals will receive an intraperitoneal injection with lipopolysaccharide (for example E. Coli 055) to elicit an immune and stress reaction	Moderate

Table 7. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

Shortly after the last behavioral test the animals will be sacrificed. The sacrificing method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood, and several other organs will also be collected for additional analyses.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size was based and calculated using standard deviations from the test with the most variation from previous experiments. The effect size was $d=1.017$, giving a minimal number of animals of 11 per group. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 11 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly besides the stress paradigm. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the experimental- or control condition. This will be to strengthen the study design by limiting variation between groups due to experimenter bias and housing influences.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

Previous studies and experience in our lab using comparable acute stress-paradigms have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. This allows us to select the minimum of 10 animals per group however, we do not want to expose each animal to more than 3 behavioral tests post-stressor/non-stressor and we aim to do 4 to 6 behavioral tests. Therefore another group of animals has to be added for the additional behavioral tests, totaling at 20 animals per group (see table 8). The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of 10 animals per group, for details see table 8.

Table 8. Maximal number of animals necessary

Animal model	Number of animals per group				Total
	No-MRI		MRI		
	Control	Stress	Control	Stress	
Orexin KO / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
Ucn1 KO / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
Ndufs4def / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
MiRNA 1 / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
MiRNA 2 / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
Total	110 / 110	110 / 110	55 / 55	55 / 55	660

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	660	Adult

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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The model we chose is one of the best validated animal models for stress-related psychopathology, and although it uses a acute stress exposure, it results in relative mild/moderate physical and psychological distress/harm compared some other stress-paradigms. The behavioral tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioral changes. Viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anesthesia (via 1.5% isoflurane inhalation) in order to minimize animal suffering. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that acute stress causes discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to

minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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.

Non applicable

Accommodation and care

F. Accommodation and care

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

[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?

G. Location where the animals procedures are performed

[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?

[] No > Continue with question I.

[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

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

During viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of 1.5% isoflurane inhalation. Otherwise no anesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

During the acute stress paradigm we will not preform anaesthesia or analgesia. When mitochondrial function will not be tested, and when perfusion will be used as a means of sacrifice, animals are first anesthetized by means of 1.5% isoflurane inhalation.

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

Stress will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, acute stress has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anaesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to acute stress, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful will be executed after the stressor to analyze differences in behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The stressors' nature is non-invasive, as well as relatively short (acute), in order to minimize physical- and mental distress, whilst still being able to induce differences between groups. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behaviour (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the acute stressors or have complications from surgery. Animals will constantly be monitored for signs of sickness, infection, excessive weight loss, or other signs of diminished wellbeing. If an animal during the experiment shows a body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or

veterinarian. Because of the non-invasive nature of the stressor, as well as the relatively short duration, and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The stressor is of a non-invasive nature, whereby the duration is not long enough that we expect severe stress to occur. The incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments. Based upon our previous experience with similar experiments with the orexin KO and Ucn1 KO mice, we expect to have less than 3% of the animals develop these criteria.

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 the animals experience from the acute stress paradigm is considered to be moderate or severe, depending on the particular stressor. The effect of the behavioral tests is expected to be mild to severe, depending on the behavioral test. The cumulative discomfort of the animals subjected to acute stress in combination with the behavioral tests is moderate or severe; for control mice this cumulative discomfort will be moderate or severe as well, as is classified is Annex VIII of the Directive 2010/63/EU.The severity of the discomfort depends on the stressor/behavioral test used, if the Porsolt swim test or electrical foot shocks are used it will be severe, if not and it the other stressor/behavioral tests the discomfort will be moderate. We expect that about 65% of the animals will experience severe discomfort and about 35% to have moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without

these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 4	Type of animal procedure Intervention experiment

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The general design of this experiment is similar compared to the former three experiments. The same animal models will be used in order to study depression susceptibility with altered protein expression, either constitutively, via miRNA intervention or through specific floxing, and/or a decreased mitochondrial function. In this experiment the animals will be treated with different antidepressants. It has been known that different antidepressants have different side effects on for example mitochondrial functioning, some antidepressants can have a positive effect whilst others have a negative effect (Klinedinst and Regenold 2015).

The specific stress model that will be used in this intervention experiment depends on the effects observed from the former three experiments in this project. The stress model will be chosen after these experiments are finished. The stressor that yields the most robust effects on behavioral, biochemical, and immunohistochemical parameters will be used in this experiment. For the different animal models, different stress paradigms might be best suited to elicit depressive behavior or robust biochemical changes. For example, one animal model will give the best results with the chronic variable stress paradigm while another might give the best result to the social defeat stress. Animals will be exposed to the stress paradigm while being treated (with either treatment or vehicle).

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, adrenal weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or mRNA in the brain, and mitochondrial function. These primary read out parameters are used because they are well-validated markers for depressive behavior, widely used, and easily measured. If these readout parameters establish a robust effect, neuroimaging will also be conducted. At the end of the experiments, behavioral data will be correlated to the biochemical data as well as neuroimaging data on brain structure and function (if neuroimaging is performed). In order to correlate biochemical, behavioral and neuroimaging data to each other the animals that undergo neuroimaging will also be behaviorally tested.

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

The aim of this experiment is to compare the genetically modified- or virally injected animals to WT littermates (non-genetically modified or control virally injected (injected with a virus containing a scrambled target sequence) target sequence respectively), by exposing them to a stressor followed by several standard behavioral tests devised to measure depression-related behavior. In addition to the stressor, in this experiment part of the animals will receive an antidepressant treatment. Two different antidepressants will be used depending on their effect on the mitochondria as

described above, as of now lithium chloride and fluoxetine would seem the bests to test at this moment. Animals will randomly be assigned to either the treatment or non-treatment group and all animals will be stressed.

Antidepressants will be given via a so called micro-osmotic pump. This is a small capsule that is inserted subcutaneous containing either an antidepressant or dissolvent. This method will be used because, although it includes surgery, it is less stressful for the animals compared to daily oral administration. At least a week before the stress paradigm, animals receive the micro-osmotic pump with either an antidepressant or dissolvent. This procedure will be done under general anesthesia (1.5 – 2% isoflurane inhalation) and the micro-osmotic pump will remain in the mouse for the duration of the experiment.

The animals that will receive a local viral injection will receive this in the same surgery as the micro-osmotic pump implantation. The viral injection is done while the animal is placed in a restraint / stereotactic apparatus, whilst the viral construct can be delivered through a thin syringe and needle.

After the surgery the animals will be subjected to either an acute stressor, a chronic stress paradigm, or social defeat stress, depending on the results from the representative experiments. The stress paradigm (and thus treatment period) that will be used is based upon the results of the previous experiments. This will also include several behavioral tests which measure behavior of the animals. For example the Porsolt swim test, tail suspension test, sucrose preference test, novelty suppressed feeing, open field test, Rotarod, splash test, and grip test. The behavioral tests that have a higher discomfort for the animals, Porsolt swim test and tail suspension test, will not be executed on the same animal. These two tests will be executed with two other milder behavioral tests. Short descriptions of these tests are included in table 9. Each behavioral test is executed once at the end of the stress paradigm.

Table 9. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

Shortly after the last behavioral test, the animals will be sacrificed. The sacrificing method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood, and several other organs will also be collected for additional analyses.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size depends on the stressor that will be used during this experiment to be determined at a later time point. If the chronic variable stress paradigm will be used the effect size is $d=0.982$, giving a minimal number of animals of 14 per group. This example is chosen because this will then also be the maximal number of animals needed in this experiment, for the other stress paradigms fewer animals will be necessary. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 11 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the one of the treatment or control conditions. This will be to strengthen the study design by limiting variation between groups due to experimenter bias and housing influences. In previous studies in our lab we observed that the subcutaneous implantation of the micro-osmotic pumps per se did not cause any mortality or extreme discomfort for the animals.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

At this point in time it is still unsure what specific antidepressants are the best option for this experiment. This is because first all three experiments must be executed and analyzed to determine the best suitable stressor per animal model. The influence of different antidepressants on mitochondrial function is a new and rapidly changing field of research, some antidepressants have a positive effect on mitochondrial function whilst others have a negative effect. As of now the best options would be to use lithium chloride which have a positive effect on the mitochondria and fluoxetine which have a negative effect on the mitochondria. A third treatment group is the control group where the micro-osmotic pumps will be loaded only with vehicle as a control. Depending on the stressor that will be chosen for this experiment, the maximum number of animals that is necessary is 14 animals per group (if chronic variable stress is used).

Previous studies and experience in our lab using comparable stress-paradigms as well as micro pump injections have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. Depending on the stressor that will be used the minimum number of animals per subgroup will be 14. Because we aim to do 4-6 behavioral tests and we do not want to expose each animal to more than 3 behavioral tests post-stressor the number of animals per has to be multiplied by two as described in the previous animal procedures,

totaling 28 animals per group (see table 3). The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of maximal 14 animals per group, for details see table 10.

Table 10. Maximal number of animals necessary

Animal model	Number of animals per group						Total
	No-MRI Stress			MRI Stress			
	Anti-1	Anti-2	Veh.	Anti-1	Anti-2	Veh.	
Orexin KO / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
Ucn1 KO / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
Ndufs4def / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
MiRNA 1 / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
MiRNA 2 / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
Total	140 / 140	140 / 140	140 / 140	70 / 70	70 / 70	70 / 70	1260

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	1260	Adult

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.

C. Re-use

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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The behavioural tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioural changes. Viral injections, micro-osmotic pump implementation, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anaesthesia (via 1.5% isoflurane inhalation) in order to minimize animal suffering. Also, 3 days post-surgery the animals will receive analgesia. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that stress and the surgery cause discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections, micro/osmotic pump placement, and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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?

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

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

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

During viral injections, telemetry sensor implementation, micro-osmotic pump insertion, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of isoflurane inhalation of 1.5%. Otherwise no anaesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

I. Other aspects compromising the welfare of the animals

I. Other aspects compromising the welfare of the animals

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

Stress will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, acute stress has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anaesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to a stressor, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful are executed after the stress paradigm to determine their behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The stress paradigms planned in this experiment are widely used in the research towards the stress response and stress related behaviors such as depressive behavior. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behaviour (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the stressors or have complications from surgery. Animals will constantly be monitered for signs of sickness, infection, excessive weight loss, or other signs of diminished wellbeing. If an animal during the experiment shows a body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or

veternarian. Because of the non-invasive nature of the stressor, as well as the relatively short duration, and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments and experience in our lab with micro-osmotic pump surgery as well as dietary interventions. We expect to have less than 3% of the animals develop these criteria.

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 the animals experience from the stress paradigm is considered severe. The effect of the behavioral tests is expected to be mild to severe, depending on the behavioral test. The cumulative discomfort of the animals in this experiment is severe (100%) as is classified is Annex VIII of the Directive 2010/63/EU.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

4.

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	Onderliggende mechanismen van stress-gerelateerde aandoeningen zoals depressie
1.2	Looptijd van het project	1-6-2016 - 1-6-2021
1.3	Trefwoorden (maximaal 5)	Stress, depressie, energiehuishouding, neuronale eiwitten

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

[X] Fundamenteel onderzoek
[] 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)	Op dit moment zijn ongeveer 350 miljoen mensen wereldwijd depressief. Depressie is de grootste oorzaak van ziekteverzuim is volgens de Wereld Gezondheid Organisatie (WHO). Depressie zorgt voor onder andere negatieve gevoelens, veranderde eetlust en verlies van plezier; daarnaast heeft het grote gevolgen voor de samenleving in de vorm van verminderde productiviteit, ziekteverzuim, en dergelijke. Ondanks jarenlang onderzoek zijn de onderliggende mechanismen van depressie nog niet voldoende bekend. Huidige medicijnen werken bij slechts 30-60% van de patiënten. De verwachting is dat depressie wordt veroorzaakt door een combinatie van omgevingsinvloeden zoals stress en bepaalde eigenschappen van de persoon, zoals veranderde eiwitwaardes in de hersenen of verlaagde energiehuishouding in het lichaam. Deze veranderde eiwitniveaus in de hersenen kunnen tot stand komen door kleine structuren genaamd microRNAs. Meer onderzoek gericht op deze theorieën kan leiden tot nieuwe vormen van behandelingrn en medicijnen waardoor een groter deel van de patiënten persoonlijker- en succesvoller behandeld kan worden.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Met dit project willen we specifieke mechanismen achter depressie ontrafelen, en op die manier een wetenschappelijke bijdrage leveren. Dit kan vervolgens de ontwikkeling van nieuwe medicijnen bevorderen, zodat patiënten beter behandeld kunnen worden. Hiermee hopen wij uiteindelijk te kunnen bijdragen aan het verlagen van de ziektelast, zowel voor de patiënt als voor de maatschappij.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Voor dit project zullen muizen worden gebruikt. Het maximale aantal muizen dat in dit project zal worden gebruikt, wordt geschat op 4260 muizen.

3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Een van de onderliggende oorzaken van depressie is stress, om dit te onderzoeken zullen de dieren gestrest moeten worden volgens algemeen geaccepteerde en veelgebruikte testen zoals een chronisch variabel stress protocol. Dit zorgt ervoor dat de dieren gestrest raken en depressief gedrag gaan vertonen. De dieren zullen ook worden geopereerd, hiervoor krijgen de dieren pijnbestrijding. Een doel is het inbrengen van een zogenaamd telemetrie apparaatje wat bijvoorbeeld activiteit en lichaamstemperatuur meet. Ook wordt er in een deel van de dieren wordt een klein pompje in het lichaam geplaatst, een zogenaamde micropomp, voor het geleidelijk toedienen van verschillende antidepressiva, zonder dat het dier hier veel last van heeft. Een ander deel van de dieren zal viraal geïnjecteerd worden om genen uit te schakelen. Wederom een ander deel van de dieren zal verdoofd worden en door middel van MRI gescand worden om effecten op hersenactiviteit te meten. Al deze voorgaande procedures zullen gepaard gaan met matige of ernstige vormen van stress en ongerief.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	De verwachte ernst van de dierproeven is matig tot ernstig. Ruwweg 32% van de dieren die in dit project worden gebruikt zal matig ongerief krijgen en 68% ernstig.
3.6	Wat is de bestemming van de dieren na afloop?	Na afloop van het experiment worden de dieren gedood om vervolgens verder onderzoek te doen op het weefsel van de dieren.

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.	Depressie is een zeer complex proces wat op dit moment alleen onderzocht kan worden in een levend dier. Er zijn naar ons weten geen goede alternatieven voor knaagdiermodellen om de aanpassing aan stress en depressief gedrag te bestuderen.

4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	De aantallen muizen die nodig zijn voor dit project zijn bepaald op basis van een diepgaand literatuuronderzoek, vergelijkbare experimenten, en statistische analyses. Dit is het minimale aantal dieren dat nodig is voor het testen van onze hypotheses. Bij gebruik van minder dieren zal het niet mogelijk zijn om verschillen tussen groepen te detecteren.
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.	Het gekozen model is een zeer geschikt en veelgebruikt diermodel voor stress gerelateerde aandoeningen zoals depressie. Het geeft ons de kans om onze resultaten met een brede basis aan literatuur te vergelijken, en geeft ook het minste ongemak voor de dieren in vergelijking met andere beschikbare modellen.
4.4	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	Wij zorgen ervoor dat alle dieren op eenzelfde manier worden gehuisvest, waarbij ze vrije toegang tot eten en drinken hebben, de hele dag door. Verder worden de dieren goed in de gaten gehouden en wordt onder andere het gewicht gemeten. Als blijkt dat een dier onnodig veel last heeft tijdens het onderzoek, dan zal in overleg met de dierverzorgers en dierenarts het dier worden geofferd.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	

5.

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer 2015-0117
2. Titel van het project: Unraveling the underlying mechanisms of depression
3. Titel van de NTS: Onderliggende mechanismen van stress-gerelateerde aandoeningen zoals depressie
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: 22-12-2015
   - ☐ aanvraag compleet
   - ☐ in vergadering besproken: 11-01-2016 en 02-02-2016
   - ☐ anderszins behandeld
   - ☐ termijnonderbreking(en) van 18-01-2016 tot 25-01-2016 en van 08-02-2016 tot 26-02-2016
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   - ☐ aanpassing aanvraag: 25-01-2016 en 26-02-2016
   - ☐ advies aan CCD: 17-03-2016
7. Eventueel horen van aanvrager
   - Datum
   - Plaats
   - Aantal aanwezige DEC-leden
   - Aanwezige (namens) aanvrager
   - Strekking van de vraag / vragen
   - Strekking van het (de) antwoord(en)
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag
8. Correspondentie met de aanvrager
   - Datum: 18-01-2015
   - Strekking van de vragen en opmerkingen:

     Project Proposal:

     -2.1 Dit is fundamenteel onderzoek naar het mechanisme waarlangs stress leidt tot depressie-gevoeligheid.

     -3.1 Het is nog onduidelijk hoe het verband is tussen de stress-hypothese (gebaseerd op een gevonden correlatie tussen stress en gevoeligheid voor depressie) en de mono-aminehypothese. De projectaanvraag behelst het aantonen van mechanismen voor de stress-hypothese, om nieuwe therapeutische targets te vinden. Waarom willen de

onderzoekers dan uiteindelijk een interventiestudie doen met oude middelen? De rationale voor een interventie met deze middelen in relatie tot de nieuwe hypotheses die getest worden ontbreekt. De commissie vindt het interventie experiment geen logisch deel uit maken van dit project en verzoekt de onderzoekers ofwel DAP-4 uit de aanvraag te verwijderen, ofwel duidelijk te beargumenteren waarom dit een logisch onderdeel is van dit project (zie ook 3.4.2).
-3.1 De relatie tussen de onderscheiden te onderzoeken factoren is niet duidelijk. Kunnen de onderzoekers duidelijker toelichten hoe de drie hypothesen onderling samenhangen?
-3.2 De onderzoekers worden verzocht hier alleen een goed afgebakende doelstelling te noemen en de haalbaarheid van die doelstelling binnen dit project toe te lichten. De tekst over de uitwerking van de doelstelling (5 verschillende diermodellen in vier verschillende experimenten) hoort thuis bij het onderdeel strategie. De onderzoekers worden verzocht één of meerdere referenties te geven waaruit blijkt dat dit project een voortzetting is van het onderzoek van deze groep.
-3.2 De onderzoekers worden verzocht de redenering om te draaien: om de mogelijk belangrijke interactie tussen de verschillende mechanismen te onderzoeken worden deze parallel onderzocht.
-3.4.2 Basaal onderzoek naar werkingsmechanismen die ten grondslag liggen aan een klinisch probleem en translationeel onderzoek naar de behandeling van dat probleem liggen doorgaans te ver uit elkaar om in één projectaanvraag te beschrijven. Wel is het denkbaar dat er in het kader van fundamenteel onderzoek enkele interventies worden gedaan ter bevestiging van het beschreven werkingsmechanisme.
-3.4.3 Waarom willen de onderzoekers het effect van drie verschillende soorten stress onderzoeken? De reden daarvoor ontbreekt in de beschrijving van de achtergrond (onderdeel 3.1).
-3.4.3 Wanneer één stressor een verandering in depressie-gevoeligheid oplevert, is het dan nog noodzakelijk om het effect van de andere stressoren te onderzoeken? Verwachten de onderzoekers niet het meest van het chronische stress model?
-3.4.3 Uit de flowchart blijkt dat dierproef 2 pas wordt gestart nadat de resultaten van dierproef 1 zijn geanalyseerd. Worden de resultaten uit dierproef 1 gebruikt om de opzet van dierproef 2 aan te passen? De onderzoekers worden verzocht duidelijker op te schrijven of er tussen de dierproeven ook go/no go momenten zijn, en welke criteria daarvoor gehanteerd zullen worden.

Description of Animal Procedures:
DAP1
-A1: Waarom worden dezelfde tests (bijvoorbeeld de FST) eerst gebruikt om chronische stress te induceren en daarna ook gebruikt om de mate van depressie verzoorzaakt door die stress te meten? Is dat wetenschappelijk wel verdedigbaar?
-A2: De onderzoekers zullen de dieren gedurende 21 dagen blootstellen aan stress en/of gedragstesten. Aan het eind van dit stress-inductie protocol zal het algemene gedrag en depressie-gerelateerd gedrag van de dieren onderzocht worden. Op dag 21 worden de dieren echter gedood. Wordt het gedrag van de dieren al tijdens het stress-inductie protocol onderzocht en zo ja, waarom? Treedt chronische stress en de depressie die daarvan het gevolg is pas na 21 dagen op, of al eerder?

-A2: Welke clusters van gedragsexperimenten maken de onderzoekers? Sommige gedragstesten zijn belastender (FST, TST) dan andere. Deze informatie is van belang om het ongerief voor een individueel dier in te kunnen schatten. Per (groep) dier(en) moet duidelijk worden gemaakt welke stressoren uit tabel 1 zij tijdens de 21 dagen ondergaan en welke gedragstesten uit tabel 2 er op volgen. Het zou overigens verhelderend zijn als de onderzoekers in de tabel voor de individuele gedragstesten het ongerief vermelden.
-A3: Willen de onderzoekers de telemetrie bij slechts 6 dieren uit een groep aanbrengen? Kunnen de 14 dieren dan nog als één groep beschouwd worden als zij niet allemaal een operatie hebben ondergaan?
-B Waarom willen de onderzoekers voor zowel de Orexin-KO als de Ucn1-KO dieren de WT dieren testen? Beide KO-stammen zijn gemaakt uit een C57BL/6J muis. Waarom wordt er slechts één groep van 14 dieren gebruikt voor het MRI experiment terwijl dit experiment precies zo zal verlopen als het niet-MRI experiment waar bij twee groepen nodig zijn, elk met verschillende gedragstesten?
-H: anesthesie tijdens perfusie ontbreekt in de opsomming. Dit staat wel bij D, refinement beschreven. (ook bij de andere DAP2)
- I: Is het ongerief t.g.v. gedragstesten wel altijd mild? FST en TST veroorzaken meer ongerief.
- K: welk percentage van de dieren zal licht ongerief ondergaan? De commissie is van mening dat de Porsolt zwemtest, de Tail suspension test, de cold stress en het gedurende een nacht huisvesten in een natte kooi ernstig ongerief voor de dieren veroorzaakt.

- Datum antwoord: 25-01-2016
- Strekking van de antwoorden:

Project Proposal:

-2.1 Wij hebben de categorie translationeel onderzoek weg gehaald zodat het alleen in de categorie fundamenteel onderzoek zit.
-3.1 In dit project wordt de interventiestudie uitgevoerd met gevalideerde en algemeen gebruikte antidepressiva. De reden hiervoor is dat deze medicijnen al langere tijd op de markt zijn waardoor dit onderzoek, als er goede resultaten worden gevonden, uiteindelijk sneller voor meer gepersonaliseerde zorg kan zorgen. De patiënten hebben er op deze manier ook sneller baat bij.
Het doel van deze interventie studie is om verschillende mechanismen te onderzoeken en te verifiëren. Het is bijvoorbeeld bekend dat sommige antidepressiva bijwerkingen op de mitochondriële functie hebben. Sommige hebben een positief effect op de mitochondriën terwijl andere een negatieve invloed hebben. In dit interventie experiment wordt bijvoorbeeld onze hypothese dat mitochondriële dysfunctie een onderliggende oorzaak van depressie is verder getest en geverifieerd in de verschillende diermodellen. Dit interventie experiment is dus een logisch vervolg van de voorgaande experimenten en er wordt onder andere de vraag beantwoord of een negatief effect van de antidepressiva op de mitochondriën een effect heeft op het gedrag van de dieren met bijvoorbeeld een mitochondrieel probleem als oorzaak van het depressieve gedrag. Of heeft alleen het antidepressivum met een positief effect een invloed op het gedrag. Hiermee worden bepaalde onderliggende mechanismen die gevonden zijn in de eerste drie experimenten aangetoond of juist afgewezen.

Ondertussen wordt er ook nog steeds veel onderzoek gedaan naar nieuwe medicijnen voor depressie, maar ook voor het beïnvloeden van mitochondriën, deze zijn echter nog niet beschikbaar. Als er in de nabije toekomst nieuwe geneesmiddelen worden uitgebracht dan zullen wij deze nieuwe middelen ook overwegen te gebruiken als deze binnen de vraagstelling vallen, zoals ook staat beschreven in de laatste zin van sectie 3.4.2 van de projectaanvraag: "… At this moment this choice cannot be made because it depends on the obtained results as well as the advances that are being made for different effects, and potentially newly discovered antidepressants".
Het volgende is toegevoegd aan de laatste paragraaf van 3.1:
"Lastly, an intervention study is utilized to test the influence of different antidepressants on depressive behavior in the stressor that gives the most robust effect in each animal model. This experiment will be done using two different classical antidepressants. These antidepressants will be chosen on for example their effect on the mitochondria. It is known that different antidepressants have a different effect on the mitochondria, some will have a positive effect whilst others have a negative effect on mitochondrial function. This intervention experiment will verify and potentially strengthen results found in first three stress experiments. Classical antidepressants are used because they are readily available and widely used, consequently if this research finds promising new results and uses for these classical antidepressants this could faster lead to more personalized health care. "
-3.1 De onderliggende basis van de drie hypotheses is dat deze alle drie invloed hebben op de stress response en op depressie susceptibiliteit zoals aangegeven in de projectaanvraag. Wat verder van belang is, is dat de drie hypotheses ook nog invloed op elkaar hebben. Zo is het bijvoorbeeld bekend dat miRNAs directe invloed hebben op heel veel verschillende eiwitten waaronder ook Ucn1 (Aschrafi et al. 2015), zoals al uitgebreid is besproken in de projectaanvraag. Verder hebben verschillende eiwitten, zoals de beschreven Ucn1 (Lawrence et al. 2004, Townsend et al. 2007, Davidson et al. 2009) en orexine (Sellayah et al. 2011), ook een direct effect op de mitochondria. Een dysregulatie van deze eiwitten kan hierdoor dus ook een directe invloed hebben op mitochondriële functie. Op deze manier kunnen de miRNAs indirect ook een invloed hebben op de mitochondriën door verschillende eiwitten te reguleren. Het is echter ook al bekend dat verschillende miRNAs een direct effect op de mitochondriën hebben (Chan et al. 2009, Chen et al. 2010). Of de mitochondriën ook een invloed hebben op de verschillende beschreven eiwitten en miRNAs is naar ons weten nog niet bekend, dit wordt dan ook in dit project onderzocht. Op deze manier komt naar boven dat deze drie hypotheses heel nauw met elkaar zijn verbonden. Hoe deze relaties echter precies zijn en welke processen upstream dan wel downstream zijn in depressie en wat voor een effect dit op de depressie symptomen heeft is echter nog onbekend en dit project draagt hieraan bij.
In dit project wordt er ook stilgestaan bij het feit dat depressie niet één ziekte is. Depressie kent veel verschillende symptomen (e.g. Criteria A in DSM V) waarbij de diagnose depressie al wordt gesteld als een aantal van de symptomen zich voordoet. Symptomen zijn bijvoorbeeld depressief gevoel, irriteerbaar, verminderde interesse of plezier in plezierige activiteiten, gewichtsverandering, verandering in eetlust, slaapstoornissen, verandering in activiteit, vermoeidheid en verlies van energie. De verschillende modellen worden dus niet alleen gebruikt omdat ze een interactie hebben met elkaar, maar ook vanwege de uiteenlopende symptomen. Een probleem bij depressie is dat de diagnose depressie al

gesteld wordt als een subset van de symptomen die worden geassocieerd met depressie zich voordoet bij een persoon. Op deze manier heeft ook niet iedereen met depressie dezelfde symptomen. Dit kan duiden op verschillende onderliggende oorzaken en mechanismen die tot de divergentie van symptomen leiden. Met de verschillende diermodellen die in dit project worden gebruikt proberen wij verschillende symptomen uit te lichten. Zo kunnen wij hiermee bijvoorbeeld onderzoeken of orexine meer invloed heeft op de verandering in het gewicht, eetlust of slaapstoornissen als symptomen, of dat de mitochondriën bijvoorbeeld meer een invloed hebben op vermoeidheid, verlies van energie of een verandering van de activiteit als symptomen van depressie. Op deze manier, samen met de drie verschillende stressoren, proberen wij te achterhalen wat de verschillende potentiële onderliggende oorzaken voor depressie zijn welke ook tot eventueel verschillende symptomen kunnen leiden.
Om dit ook te verduidelijken in de projectaanvraag is de volgende tekst toegevoegd aan het einde van sectie 3.1:
"As described above, all three hypotheses have an influence on the stress response as well as on depression susceptibility. However, it seems that they also interact with each other. For example, miRNAs have a direct influence on different proteins such as Ucn1 (Aschrafi et al. 2015). Also the different described proteins such as Ucn1 (Lawrence et al. 2004, Townsend et al. 2007, Davidson et al. 2009) and orexin (Sellayah et al. 2011) have a direct influence on the mitochondria. A dysregulation of these proteins could also have a direct influence on mitochondrial functioning. In this way miRNAs can also have an indirect influence on the mitochondria by regulating or dysregulating different proteins. However, it has also been found that different miRNAs have a direct effect on the mitochondria (Chan et al. 2009, Chen et al. 2010). If this is also the other way around, that the mitochondria have a direct effect on the described proteins or miRNAs is unclear, this will also be investigated in this project. It seems that these three hypotheses are tightly linked, however what the mechanisms are, what processes are upstream or downstream, and what their influence is on depression symptoms are, is unknown. This project will contribute to unraveling this.
In this project we will also investigate the fact that depression is not only one disease. Depression has many different symptoms (e.g. Criteria A in DSM V) where only 5 out of 9 symptoms are required for the diagnosis depression. The different symptoms are depressed mood or irritable, decreased interests or pleasure, significant weight change or change in appetite, change in sleep, change in activity, fatigue or loss of energy, guilt/worthlessness, concentration, and suicidality. The different animal models will not only be used because they have interacting mechanisms, but also because they can shed light on potential different mechanisms underlying different symptoms of depression. Because only 5 out of 9 symptoms must be present to diagnose depression, there are many different combinations possible resulting in the fact that not everyone have the same symptoms. This can point towards different mechanisms that we can potentially investigate with the different animal models. For example, this way we can investigate if orexin influences the change in weight, appetite or sleep as depression symptoms, or that the mitochondria are underlying the fatigue, loss of energy or a change of activity. Together with the three different stress models we try to elucidate the potential different underlying causes of depression that can explain the different symptoms with different people."

-3.2 Er zijn een aantal kleine veranderingen gedaan in de tekst van de doelstelling (dikgedrukte tekst) en de tekst over de uitwerking van de doelstelling is verplaatst naar het onderdeel strategie.

"This project aims to provide several important novel insights in the etiology and biological underpinnings of stress related disorders such as depression using various translational animal models. For example, the involvement of mitochondria in the susceptibility in depression, the involvement of certain microRNAs in the pathophysiology of stress related disorders, as well as the involvement of different peptides such as Ucn1, orexin, and CRF in the stress response and depression susceptibility. **In addition to this, possible interactions between these different biological underpinnings will also be investigated as well as how much these could contribute to the pathology of various Category A depression symptoms (DSM V) like fatigue, sleep problems, psychomotor retardation, etc.**. The use of novel hypotheses, as outlined in section 3.1, in combination with well-validated models for stress-related psychological diseases with the focus on depression, will help us better understand the underlying mechanisms of stress related psychiatric diseases **as well as a better understanding of the various category A symptoms of depression**. Consequently, it could also give new insights in identifying novel therapeutic targets and treatment strategies of major depression. In addition, important data will be acquired to better understand fundamental mechanisms contributing to stress adaptation, and consequently will increase our insight into neuronal processes that may underlie unsuccessful adaptation to stress and depression susceptibility. Ultimately, this may lead to the development of new treatment targets **or the re-categorization of current treatments which is investigated in experiment 4**. Because the project examines various mechanism in parallel, it also offers the possibility to investigate the interaction between the above detailed mechanisms in depression vulnerability. We will be able to identify different behavioral, physiological, and endocrine aspects of depression that can be correlated/compared to similar parameters in humans giving this project a great translational value."

Enkele referenties zijn toegevoegd die aangeven dat dit project een voortzetting is van onze groep is. In de laatste zin van de laatste paragraaf (vetgedrukt is toegevoegd):

"…This project will be a continuation of the research that has already been performed in this lab towards unraveling the underlying mechanisms of depression. **Previous experiments encompass miRNA 326 and Ucn1 (Aschrafi et al. 2015), orexin and stress ([redacted]), Ucn1 and depression (Kozicz 2007, Spencer et al. 2012), as well as mitochondrial function and depression ([redacted])**."

-3.2 De redenering is omgedraaid. In sectie 3.2 staat nu het volgende:

"… In order to investigate the interaction between the above detailed mechanisms in depression vulnerability, the various mechanisms mentioned in the background are examined in parallel. …"

-3.4.2 Wij zijn het hiermee eens, naar aanleiding van het eerste punt is ook de translationeel categorie weg gehaald. In dit project wordt de interventiestudie gebruikt voor het testen van de onderliggende mechanismen. Als bijvoorbeeld een lagere mitochondriële activiteit de oorzaak is van depressie symptomen bij een bepaalde patiënt, kan een antidepressivum waarvan bekend is dat deze een negatieve invloed heeft op de mitochondria wel een positief effect hebben op de depressie symptomen. Met dit interventie experiment kunnen wij

bepaalde onderliggende werkingsmechanismen aantonen of juist afwijzen zoals beschreven in antwoord op de bovenstaande eerste vraag over 3.1.
-3.4.3 Het is al bekend dat stress een invloed heeft op het krijgen van depressie. Het is ook bekend dat verschillende soorten stress verschillende effecten hebben op verschillende individuen en dat er verschillende onderliggende mechanisme worden geactiveerd (Lamb 1979, Briski and Gillen 2001, Kavushansky et al. 2009). Het is dus belangrijk om verschillende stressoren toe te passen in de diermodellen die wij hier gebruiken om deze verschillen te onderzoeken. De twee chronische stressoren die in dit project worden gegeven belichten andere aspecten van stress en de invloed op depressie. De ene stressor focust zich meer op de fysieke kant en onvoorspelbaarheid van stress (chronische stress) terwijl de andere zich meer focust op de psychologische kant van stress (sociale stress) (zoals beschreven in 3.4.2). Ook is het gebruik van verschillende stressoren belangrijk omdat depressie niet één ziekte is, zoals al in een eerder antwoord hierboven aangegeven. Voor de diagnose depressie zijn maar 5 van de 9 categorie A symptomen nodig. Met deze twee verschillende chronische stressoren verwachten wij bij de verschillende diermodellen andere uitkomsten. De acute stress draagt hier ook aan bij, deze is noodzakelijk om te bepalen of er in de verschillende diermodellen een verschil is in de initiële stress response wat de effecten van de chronische stressoren zou kunnen verklaren.
Om deze redenatie duidelijker naar voren te laten komen in de projectaanvraag is in onderdeel 3.1 het volgende toegevoegd:
“This project also utilizes three different kinds of stressors, namely chronic variable stress, chronic social defeat stress, and acute stress. These three stressors are necessary because it is known that physical and psychological stressor have other effects on different individuals and on underlying mechanisms (Lamb 1979, Briski and Gillen 2001, Kavushansky et al. 2009). The chronic variable stress focusses more on the physical and unpredictable component of the stressor while the social defeat stress focusses more on the psychological component of stress. To determine if the initial stress response is different between WTs and the different animal models in this project an acute stressor is used.”
-3.4.3 Zoals bij de hierboven beantwoorde vraag uitgebreid is uitgelicht, is het noodzakelijk om verschillende stressoren te gebruiken, een meer fysieke stressor en een meer psychologische stressor. Hiernaast wordt dan de acute stressor gebruikt voor inzicht in de initiële stress response bij de verschillende diermodellen.
Wij verwachten niet per se het meeste effect van het chronische stress model, het sociale stress model is ook een erg goed beschreven en robuust model voor het onderzoek naar depressie gevoeligheid. Daarnaast is deze stressor van een andere aard dan de chronische variabele stress waarvoor wij dus ook andere uitkomsten in bijvoorbeeld categorie A gedrag verwachten bij de verschillende diermodellen. Dit geeft dan weer meer inzicht in de verschillende potentiële onderliggende mechanismen.
-3.4.3 Tussen dierproeven 1, 2 en 3 zitten geen go/no go momenten. Dit omdat enerzijds alle stressoren toegepast dienen te worden om een zo compleet mogelijk beeld te krijgen binnen dit project, en anderzijds omdat de verschillende dierproeven niet op elkaar gebaseerd zijn. De opzet van de verschillende dierproeven is wel hetzelfde, maar niet afhankelijk van de vorige dierproef en de resultaten hebben geen directe invloed op.
Om dit duidelijker te maken in het project is de volgende tekst toegevoegd (dikgedrukt is toegevoegd:

“The four proposed animal procedures will be executed in succession with each other (see figure 1). This flowchart will be used for each of the five proposed animal models (orexin KO, Ucn1 KO, Ndufs4 deficient, and two virally injected groups). **Between the different animal procedures there will not be a go/no-go evaluation moment.**”

Description of Animal Procedures:
DAP1 Als bijvoorbeeld de FST wordt gebruikt als stressor tijdens de chronische stress zal dit aan het begin zijn van het protocol. Deze test zal dan zowel dienen als stressor en als gedragstest, en dient ervoor om een baselinewaarde voor elk dier te bepalen. Op deze manier kunnen wij het gedrag van deze dieren ook longitudinaal volgen en vergelijken voor en na het stressprotocol binnen een individu. Op deze manier dient deze gedragstest als twee functies.
-A2: De chronische stress treedt geleidelijk op en uit voorgaand onderzoek blijkt dat 21 dagen voldoende is om depressief gedrag te induceren. Dit betekent niet dat de dieren op dag 20 geen depressief gedrag vertonen en op dag 21 wel, net als de chronische stress is dit een geleidelijk proces. Het gedrag zal niet abrupt veranderen op de laatste dag. Om deze reden zal bekeken worden of op de laatste paar dagen al een gedragsexperiment kan worden geïmplementeerd dat ook stressvol is voor de dieren. Een voorbeeld is om een ‘novelty suppressed feeding’ test uit te voeren. Om deze test uit te voeren moeten de dieren de nacht van tevoren niet hebben gegeten (overnacht zonder voer) wat ook een stressor is. Op deze manier wordt het dier gestrest en kan er tegelijkertijd een gedragstest worden uitgevoerd tegen het einde van het protocol waardoor er op dag 21 niet drie testen achter elkaar uitgevoerd hoeven te worden wat voor meer ongerief zou hebben gezorgd. Aan het begin van het stressprotocol kan ook bijvoorbeeld een FST worden uitgevoerd als baselinemeting, zie ook het antwoord op de vraag hierboven.
-A2: Het is correct dat in dit project verschillende gedragstesten zitten met een verschillende belasting op het ongerief van de dieren. Hiermee wordt rekening gehouden tijdens het plannen van de experimenten. De clusters van gedragsexperimenten zullen zo gemaakt worden dat zwaar belastende gedragstesten, zoals de FST en TST, niet beide op een dier wordt toegepast. Een zwaar belastende gedragstest zal altijd worden uitgevoerd met twee mildere gedragstesten. Aan het einde van het stressprotocol en de gedragstesten is het ongerief voor de dieren die het chronische variabele stress proton ondergaan cumulatief ernstig. Dit is echter ook noodzakelijk omdat depressie ook is geassocieerd met ernstige stress, milde stress resulteert niet in depressie.
De volgende tekst is toegevoegd aan de DAP (dikgedrukt is toegevoegd):
“At the end of the stress paradigm general and depressive related behavior of the animals will be analyzed with several different behavioral experiments. For example the Porsolt swim test, tail suspension test, sucrose preference test, novelty suppressed feeing, open field test, Rotarod, splash test, and grip test. **The behavioral tests that have a higher discomfort for the animals, Porsolt swim test and tail suspension test, will not be executed on the same animal. These two tests will be executed with two other milder behavioral tests. …"**
De stressoren die in tabel 1 staan zullen allemaal worden gebruikt in het chronisch variabel stress protocol. Dit is noodzakelijk om de onvoorspelbaarheid van de stressoren te waarborgen. Als er minder verschillende stressoren worden toegepast dan zullen de dieren er gemakkelijker aan kunnen wennen en potentieel anticiperen waardoor het protocol

minder effectief is (Willner 2005). Alleen de gedragstesten die genoemd zijn in tabel zullen niet altijd gebruikt worden.
De volgende tekst is toegevoegd aan de DAP ter verduidelijking (dikgedrukt is toegevoegd):
"… Each animal in the stress group receives the same stressors and these stressors will be presented in a randomized order where each stressor **mentioned in table 1** is presented an equal number of times **(except for the behavioral tests mentioned in the table)**. …"
Verder is het ongerief van de individuele stressoren en gedragstesten aan de tabellen toegevoegd.
-A3: Dit is een fout van onze kant, deze zin had er niet meer in moeten staan en is dus ook verwijderd. Onze initiële opzet was dat een aparte groep zou worden geïmplementeerd met een telemetrie sensor, maar van dit concept zijn wij afgestapt. Nu zullen alle dieren worden geopereerd.
-B Het is inderdaad zo dat beide stammen een C57BL/6J achtergrond hebben, echter hebben wij voor de drie beschreven genetisch gemodificeerde diermodellen een heterozygote fok. Dit is voornamelijk om ervoor te zorgen dat het gedrag van de moeder naar de pups toe gelijk is bij de WT en KO dieren. Het is namelijk bekend dat bij een homozygote fok de moeders van WT en KO muizen anders voor de pups zorgen wat veel invloed heeft op stress en depressie onderzoek. Omdat de heterozygote orexine en Ucn1 moeders een verschillend genotype hebben en dus potentieel verschillend gedrag richting de pups hebben zal in dit project beide WT en KO dieren moeten testen van de orexine KO en Ucn1 KO dieren.
De reden dat er maar één groep dieren wordt gebruikt voor het MRI experiment is omdat wij tijdens het go/no-go moment evalueren welke gedragstesten het meest robuuste resultaat geven. Hieruit kiezen wij dan maar één cluster van drie gedragstesten om het aantal dieren te verminderen. Aan het einde van sectie 2B staat dit ook beschreven: "If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of 14 animals per group, for details see table 3."
In sectie 2A is dit ook aangepast (dikgedrukt is toegevoegd):
Sectie 2A tweede stuk begin laatste alinea: "If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. **Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. …**"
-H: In de opsomming hebben wij de anesthesie tijdens de perfusie meegenomen (dikgedrukt is toegevoegd):
"During viral injections, telemetry sensor implementation, **perfusion as a method of sacrifice**, or MRI experiments animals will be anesthetized by means of 1.5% isoflurane inhalation. …"
- I: Wij zijn het hiermee eens, de twee genoemde gedragstesten geven meer stress aan de dieren. Dit hebben wij nu veranderd:
"… Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. …"

- K: Wij zijn het niet eens met de commissie dat de Porsolt zwemtest en tail suspension test ernstig ongerief veroorzaken. Wij zijn van mening dat deze gedragstesten matig ongerief veroorzaken bij de dieren door de korte tijdsduur van de test. Dit hebben wij ook gevonden in een document voor de ongeriefscores van de DEC van Groningen. Over de ernstigheid van de cold stress en overnacht huisvesten in een kooi met natte bedding zijn wij het wel eens met de commissie, dit is dan ook veranderd naar ernstig ongerief.
  Uiteindelijk hebben wij de ongeriefscores herbeoordeeld, aan het einde van het chronische stress protocol en de gedragstesten hebben de dieren ernstig ongerief. De dieren die niet het chronische stress protocol volgen zullen matig ongerief hebben aan het einde.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

- Datum: 08-02-2016
- Strekking van de vragen:
  Description of Animal Procedures:
  -De Porsolt zwemtest geeft naar het oordeel van de commissie bij muizen, die veel minder goed kunnen zwemmen dan ratten, ernstig ongerief. Niet de duur van de test, maar het feit dat er sprake is van uitputting en de angst om te verdrinken bepalen in dit geval het ongerief. De commissie weet zich daarin gesteund door bijlage VIII van de Europese richtlijn 2010/63/EU, deel III onderdeel 3. m). De onderzoekers worden verzocht dit aan te passen in de projectaanvraag.
  Datum antwoord: 11-02-2016 en 25-02-2016 (dikgedrukte tekst)
- Strekking van de antwoorden:
  Description of Animal Procedures:
  - In de beschrijving van de dierprocedures is het ongerief van het gebruik van de Porsolt zwemtest veranderd van matig naar ernstig ongerief. Dit is gedaan in alle tabellen en tekstdelen.
  In de classificatie van de ernstigheid is de volgende tekst ook nog toegevoegd aan de eerste drie experimenten:
  DAP1:
  The discomfort severity of the control mice depends on the cluster of behavioral tests, if the cluster includes the Porsolt swim test it will be severe **(50% of the control animals),** if not and it includes the tail suspension test it will be moderate **(50% of the animals). In total, 75% of the animals will experience severe discomfort and 25% of the animals will experience moderate discomfort.**
  DAP2:
  The discomfort severity which will face C57BL/6J mice depends on the cluster of behavioral tests. **The mice that will face the C57BL/6J mice and receive the Porsolt swim test will have severe discomfort (50%) while the animals that face the C57BL/6J mice and do not receive the Porsolt swim test will have moderate discomfort (50%). In total, 37.5% of the animals (including both residents and intruders) will experience severe discomfort and 62.5% of the animals will experience moderate discomfort.**
  DAP3:
  The severity of the discomfort depends on the stressor/behavioral test used, if the Porsolt swim test or electrical foot shocks are used it will be severe, if not and it the other stressor/behavioral tests the discomfort will be moderate. **We expect that about 65% of the animals will experience severe discomfort and about 35% to have moderate discomfort.**

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

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

B. Beoordeling (adviesvraag en behandeling)

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

C. Beoordeling (inhoud):

1. Het project is:
   - uit wetenschappelijk oogpunt verantwoord

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.
3. De DEC onderschrijft het belang van de doelstelling, namelijk 'to provide important novel insights in the etiology and biological underpinnings of stress related disorders such as depression using various translational animal models.' De onderzoekers richten zich daarbij op de bijdrage van mitochondriën, van bepaalde microRNAs en van de peptides Ucn1, orexin en CRF aan de gevoeligheid voor depressie gemeten op gedrags-, fysiologisch en hormonaal niveau. De onderzoekers hebben in antwoord op vragen van de DEC voldoende aannemelijk gemaakt dat de hypotheses die aan het onderzoek ten grondslag liggen (over de rol van de mitochondriën en over de rol van bepaalde microRNAs en de peptides Ucn1, orexin en CRF bij gevoeligheid voor depressie) met elkaar samenhangen. De te behalen resultaten zullen duidelijk maken of deze factoren afzonderlijk of door interactie met elkaar van invloed zijn op het ontstaan van depressie na blootstelling aan stress, en of zij samenhangen met specifieke symptomen van depressie bij muizen. Voorts levert dit onderzoek aanknopingspunten op voor verder onderzoek naar differentiële toepassing van antidepressiva voor subgroepen van patiënten. Maatschappelijk is dit onderzoek van belang, omdat veel mensen gedurende hun leven een depressie doormaken. De WHO rapporteert dat depressie op dit moment wereldwijd de belangrijkste ziekteoorzaak is. Depressie heeft niet alleen een grote impact op de patiënt zelf, maar ook op zijn of haar familie. Aangezien depressie op alle leeftijden kan optreden is er ook een grote economische impact op de maatschappij. De DEC acht meer inzicht in het ontstaan van depressie na blootstelling aan stress en effectievere therapie voor depressie van substantieel belang, gezien de omvang van het probleem en de grote groep patiënten die geen baat heeft bij de huidige behandeling met antidepressiva.
4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Deze groep heeft veel ervaring in dit onderzoeksveld en met de

voorgestelde dierproeven. De gekozen aanpak leidt tot meer inzicht in neuronale processen die mogelijk ten grondslag liggen aan onvoldoende aanpassing aan stress en gevoeligheid voor depressie.

5. Er is geen sprake van bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren.
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd. Het ongerief wordt hoofdzakelijk bepaald door de stress waaraan de dieren worden blootgesteld. De DEC schat het ongerief als gevolg van de meeste gedragstesten en enkele stressoren, en het imagen onder anesthesie waarna het dier wordt gedood in als licht. Het ongerief als gevolg van de meeste stressoren en de operatie voor het aanbrengen van telemetrie-apparatuur of een micro-osmotische pomp waarbij sommige dieren tevens een injectie in de hersenen krijgen schat de commissie in als matig. De DEC schat het ongerief als gevolg van de forced swim test, de koudestress, het gedurende de nacht verblijven in een natte kooi en de onvermijdbare elektrische schokken in als ernstig. Het cumulatief ongerief voor de muizen in de beschreven vergunningaanvraag is dus juist ingeschat als matig voor 32% van de dieren en ernstig voor 68% van de dieren.
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.** De doelstelling van het project kan niet gerealiseerd worden zonder proefdieren of door gebruik van minder complexe diersoorten. Het gedrag na blootstelling aan stress kan alleen goed bestudeerd worden bij proefdieren. Het gedragsrepertoire van de muis is voldoende complex om de translationele waarde van dit onderzoek te waarborgen. De onderdelen van het onderzoek die zonder proefdieren uitgevoerd kunnen worden, zijn al uitgevoerd of zullen *in vitro* uitgevoerd worden. Voor de resterende onderzoeksvragen is het gebruik van proefdieren noodzakelijk.
8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de **vermindering** van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat en proportioneel ten opzichte van de gekozen strategie en de looptijd. De DEC is het eens met het beschreven onderzoeksmodel en de onderbouwing van het aantal benodigde dieren. Door de stapsgewijze aanpak waarin de resultaten uit eerdere proeven worden gebruikt voor het design van vervolgexperimenten wordt onnodig gebruik van proefdieren voorkomen. De DEC is van oordeel dat het project kan worden uitgevoerd met maximaal 4260 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. Dagelijkse controles van de dieren zorgen ervoor dat bij onverwacht optredend ongerief tijdig kan worden ingegrepen. Operaties aan de dieren worden gecombineerd zodat zij slechts éénmaal hoeven te herstellen van de narcose. Depressie is een ernstige aandoening die kan ontstaan na blootstelling aan ernstige stress. Mildere vormen van stress leiden niet tot depressie, waardoor deze ernstige stress onvermijdelijk is om het effect van stress op de gevoeligheid voor depressie te kunnen onderzoeken. De DEC is ervan overtuigd dat de dierproeven verder zo humaan mogelijk worden uitgevoerd.
   Er is geen sprake van belangwekkende milieueffecten.
10. De niet-technische samenvatting is een evenwichtige weergave van het project, zelfstandig leesbaar, beknopt en begrijpelijk geformuleerd.

D. Ethische afweging

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

Met dit onderzoek worden belangrijke wetenschappelijke inzichten verworven in neuronale processen die mogelijk ten grondslag liggen aan het verschijnsel dat onvoldoende aanpassing aan stress de gevoeligheid voor depressie lijkt te verhogen. Voorts wordt duidelijk of bepaalde processen samenhangen met specifieke symptomen van depressie. Het is aannemelijk dat de medicatie van depressieve mensen hierdoor beter kan worden afgestemd op het individuele ziekteproces. Het belang van meer inzicht in de factoren die bijdragen aan de gevoeligheid voor depressie na blootstelling aan stress acht de DEC substantieel, omdat veel mensen gedurende hun leven een depressie zullen doormaken en de huidige behandeling met antidepressiva bij veel mensen onvoldoende effect heeft.

Tegenover dit substantiële belang staat het gegeven dat 32% van de dieren matig ongerief en 68% van de dieren ernstig ongerief zullen ondervinden als gevolg van de stress waaraan zij worden blootgesteld in combinatie met de benodigde handelingen. De commissie realiseert zich dat het hier gaat om grote aantallen muizen die ernstig ongerief zullen ondervinden door de stressoren waaraan zij worden blootgesteld. Er is echter geen andere manier om de onderzoeksvraag goed te kunnen onderzoeken, en in het licht van de omvang en de ernst van het probleem dat wordt onderzocht acht de commissie deze experimenten aanvaardbaar. De commissie is er van overtuigd dat bij de dierproeven adequaat invulling gegeven zal worden aan de vereisten op het gebied van de vervanging, vermindering en/of verfijning van dierproeven. Het gebruik van de dieren en het daarbij optredende ongerief is onvermijdelijk, wil men de doelstellingen kunnen realiseren.

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

E. Advies

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

2. Het uitgebrachte advies is gebaseerd op consensus.

Centrale Commissie Dierproeven

6.

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Katholieke Universiteit Nijmegen
[redacted]
Postbus 9101
6500 HB [redacted] NIJMEGEN

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

Onze referentie
Aanvraagnummer
AVD103002016481

Bijlagen
2

Datum 18 maart 2016
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted]

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 18 maart 2016.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002016481. Gebruik dit nummer wanneer u contact met de CCD opneemt.

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

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

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

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

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

Gegevens plaatsvervangende verantwoordelijke onderzoeker

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

Gegevens verantwoordelijke uitvoering proces

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

Gegevens gemachtigde

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

Over uw aanvraag

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

Over uw project

Geplande startdatum:	1 juni 2016
Geplande einddatum:	1 juni 2021
Titel project:	Unraveling the underlying mechanisms of depression
Titel niet-technische samenvatting:	Onderliggende mechanismen van stress gerelateerde aandoeningen zoals depressie
Naam DEC:	RU Dec
Postadres DEC:	Postbus 9101, 6500 HB Nijmegen [redacted]
E-mailadres DEC:	[redacted]

Betaalgegevens

De leges bedragen:	€ 1.584,-
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] Melding Machtiging [x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Nijmegen
Datum:	18 maart 2016

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Instantie voor Dierenwelzijn
Postbus 9101
6500 HB [redacted] NIJMEGEN

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

Onze referentie
Aanvraagnummer
AVD103002016481

Bijlagen
2

Datum 18 maart 2016
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 18 maart 2016
Vervaldatum: 17 april 2016
Factuurnummer: 16700481
Ordernummer: 040823-461220/ 2015-0129/ [redacted]

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD103002016481	€ 1.584,00

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

Centrale Commissie Dierproeven

7.

> Retouradres Postbus 20401 2500 EK Den Haag

Radboud Universiteit Nijmegen
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[redacted]
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Centrale Commissie Dierproeven

Postbus 20401
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T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD103002016481

Uw referentie

Bijlagen
1

Datum 19 april 2016
Betreft Aanvulling Aanvraag projectvergunning dierproeven

Geachte [redacted]

Op 18 maart 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Unraveling the underlying mechanisms of depression" met aanvraagnummer AVD103002016481. In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In deze brief leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

1) U beschrijft in uw aanvraag meerdere gedragstesten te willen gebruiken om de depressie in muizen te meten/te onderzoeken. U geeft aan dat de groepen die voor het MRI onderzoek worden gebruikt alleen met de meest veelbelovende gedragstesten worden getest. In het kader van vermindering en verfijning, zouden we u willen vragen of in de dierproeven 3.4.4.2 - 3.4.4.4 ook alleen de meest betrouwbare testen uit dierproef 3.4.4.1 gebruikt kunnen worden. Dat zou voorkomen dat extra groepen dieren nodig zijn voor testen die in hetzelfde model geen positieve resultaten laten zien. U geeft aan dat de dierproeven niet parallel worden uitgevoerd, dus dit zou in uw planning haalbaar zijn.

2) In de bijlage dierproeven 3.4.4.2 geeft u aan één 'residentmuis' per testmuis te willen gebruiken, dus net zoveel residentmuizen als testmuizen nodig te hebben. Kunt u onderbouwen waarom u de residentmuizen niet kunt hergebruiken, verwacht u dat alle 720 testmuizen tegelijkertijd worden getest? In het kader van mogelijkheden tot vermindering verzoeken we u om hierop te reageren.

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

Om u aanvraag in de eerstkomende CCD vergadering te kunnen bespreken ontvangen we graag uw antwoord uiterlijk **maandag 25 april 2016**.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen.

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

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.zbo-ccd.nl
- Of bel met ons: 0900 28 000 28 (10 ct/min).

1 Uw gegevens

1.1 Vul de gegevens in.

Naam aanvrager			
Postcode		Huisnummer	

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

Aanvraagnummer	

2 Bijlagen

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

☐	
☐	
☐	

3 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Naam	
Datum	- - 20
Handtekening	

1) U beschrijft in uw aanvraag meerdere gedragstesten te willen gebruiken om de depressie in muizen te meten/te onderzoeken. U geeft aan dat de groepen die voor het MRI onderzoek worden gebruikt alleen met de meest veelbelovende gedragstesten worden getest. In het kader van vermindering en verfijning, zouden we u willen vragen of in de dierproeven 3.4.4.2 - 3.4.4.4 ook alleen de meest betrouwbare testen uit dierproef 3.4.4.1 gebruikt kunnen worden. Dat zou voorkomen dat extra groepen dieren nodig zijn voor testen die in hetzelfde model geen positieve resultaten laten zien. U geeft aan dat de dierproeven niet parallel worden uitgevoerd, dus dit zou in uw planning haalbaar zijn.

Onze hypothese is dat depressie niet een homogene ziekte is, maar een samenspel van verschillende symptomen welke zeer waarschijnlijk ontstaan door verschillende onderliggende biologische processen. Zoals ook beschreven in sectie 3.1 van het project voorstel is het zo dat niet iedereen met depressie dezelfde symptomen heeft. Verder verwachten wij dat verschillende stressoren verschillende biologische processen in het lichaam activeren die kunnen leiden tot depressie. De verschillende gedragstesten die in dit project worden gebruikt onderzoeken de verschillende symptomen van depressie. In de dierproeven 3.4.4.1 – 3.4.4.4 testen wij verschillende onderliggende biologische processen, hierdoor verwachten wij dat in de verschillende dierproeven een verschil in gedrag is te zien. Het kan bijvoorbeeld zo zijn dat in dierproef 3.4.4.1 een effect wordt gevonden in de Porsolt zwemtest, terwijl voor dierproef 3.4.4.2 de Porsolt zwemtest wellicht geen verschil laat zien. Dit kunnen wij echter niet van tevoren vaststellen, dit is namelijk een van de innovatieve aspecten die wij in dit project onderzoeken. Om deze reden is de exclusie van verschillende gedragsexperimenten op basis van dierproef 3.4.4.1 niet mogelijk en is het van belang dat alle gedragstesten worden uitgevoerd in de verschillende dierexperimenten. Het klopt dat wij voor de MRI alleen de gedragstesten gebruiken die het meeste effect laten zien, dit is dus mogelijk omdat in dezelfde dierproef dezelfde onderliggende biologische processen worden onderzocht.

Voor dierproef 3.4.4.4 is er wel een mogelijkheid om te bekijken welke gedragstesten worden gebruikt. Als blijkt dat er maar 3 gedragstesten interessant zijn om uit te voeren dan hebben wij de helft minder dieren nodig. Als echter blijkt dat meer dan 3 verschillende gedragstesten belangrijk zijn om uit te voeren dan zal er als nog de originele hoeveelheid dieren nodig zijn.

Dit hebben wij nu in een go/no-go moment verwerkt. Hierdoor veranderd het maximale aantal van de aanvraag echter niet. De tekst in groen is toegevoegd aan 3.4.3 van het project voorstel: "… The paradigm that gave the most reliable and robust results will be used for the intervention experiment to minimize the number of animals used. Also, if less than three behavioral tests are interesting to investigate in this experiment we will use only one cluster of behavioral tests and not two clusters as in the previous experiments, thus reducing the number of animals used."

En het volgende is toegevoegd (in groen) aan de sectie B van DAP 4: "… Depending on the stressor that will be used the minimum number of animals per subgroup will be 14. Depending on the number of behavioral tests that yielded interesting results from the experiment that this intervention experiment is based upon, we might need only one cluster of animals. If 3 or fewer behavioral tests are interesting to investigate only one cluster of animals will be used, if 4 or more behavioral tests will be executed we will have to multiply the number of animals by two as described in the previous animal procedures, totaling 28 animals per group (see table 10). This is because we do not want to expose each animal to more than 3 behavioral tests post-stressor. …"

2) In de bijlage dierproeven 3.4.4.2 geeft u aan één 'residentmuis' per testmuis te willen gebruiken, dus net zoveel residentmuizen als testmuizen nodig te hebben. Kunt u onderbouwen waarom u de residentmuizen niet kunt hergebruiken, verwacht u dat alle 720 testmuizen tegelijkertijd worden getest? In het kader van mogelijkheden tot vermindering verzoeken we u om hierop te reageren.

Wij zijn het eens met de Centrale Commissie Dierproeven dat in het kader van vermindering de residentmuizen kunnen hergebruiken. Van tevoren kunnen wij echter niet voorspellen hoe de residentiemuizen zullen reageren op een langere periode van de introductie van testmuizen. Wij denken wel dat elke residentiemuis voor twee experimenten kan worden gebruikt, hierdoor kan het aantal residentmuizen worden gehalveerd naar 360. Dit kom neer op een totaal aantal dieren van 1080 in DAP 2.

Omdat wij niet zeker zijn hoe de residentmuizen zullen reageren op meerdere weken gebruikte te worden voor het experiment kunnen wij op dit moment het aantal muizen niet nog verder naar beneden brengen.

In de dierexperimentele procedure 2 hebben wij het aantal dieren aangepast. Ook in de niet technische samenvatting hebben wij het aantal dieren aangepast van in totaal 4260 naar 3900 muizen.

De volgende tekst is toegevoegd aan DAP2 sectie B1: "**The number of residents required for this experiment is half of the number of intruders described above (see table 5). Only half of the animals is necessary because we expect that we can use the resident mice in two separate experiments. However, we will ensure that** each intrudes see a different resident every time to avoid recognition between the animals."

9.

Form
Project proposal

- This form should be used to write the project propsal of animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed
- For more information on the project proposal, see our website(www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	Unraveling the underlying mechanisms of depression

2 Categories

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

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

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

3 General description of the project

3.1 Background

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

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

Currently, depression is thought to be caused by an interaction from the environment (stress) and (genetic) predispositions of the individual. Whilst the exact mechanism is not known, it is known that there is a higher risk of developing depression when more stressful life events have occurred (Brown & Harris, 1978, Nemeroff and Vale 2005). Numerous human and animal research show that adverse live events or stress are undoubtedly one of the major risk factors for the development of depression (McEwen 2003, De Kloet et al. 2005, Nemeroff and Vale 2005, Joëls and Baram 2009, Schmidt 2011, Morava and Kozicz 2013). A stressor is something that challenges the individual and is in conflict with the individual's homeostasis. It can be either external or internal, and physical or psychological (Chrousos and Gold 1992, McEwen 2003, De Kloet et al. 2005). In order to maintain homeostasis, the individual must challenge the stressor; one way to do this is by generating the so-called stress response. The best-known and characterized stress response system is the hypothalamic-pituitary-adrenal (HPA)-axis. In this system, upon a challenge, corticotropin-releasing factor (CRF) is released, which via the pituitary gland activates the adrenal glands to release corticosteroids. The released mineralo- and glucocorticoids mediate various important physiological adaptive processes. These adaptive processes involve the recruitment of multiple brain areas and complex brain networks (De Kloet et al. 2005, Pittenger and Duman 2008, Joëls and Baram 2009, Price and Drevets 2012, McEwen et al. 2015). Depending on the stressor, different parts of these networks are recruited that will coordinate different physiological, neuroendocrine, and behavioral aspects of the stress response (Joëls and Baram 2009, Krishnan and Nestler 2010, Morava and Kozicz 2013). If one or more of the stress responsive systems are not functioning properly, the different physiological processes to adequately cope with the stressor cannot be activated properly and the stressor cannot be handled in an adaptive manner increasing the risk to develop depression (McEwen 1998, Brown et al. 2004, De Kloet et al. 2005, Joëls and Baram 2009, Krishnan and Nestler 2010). For example, for over more than two decades it has been known that there is a direct relationship between depression and alterations in the HPA-axis and the CRF system (Nemeroff et al. 1984, Banki et al. 1987, Raadsheer et al. 1994, Bale and Vale 2004, Merali et al. 2004, De Kloet et al. 2005, Pariante and Lightman 2008).

One of the best-known hypotheses for the underlying cause of depression is the monoamine hypothesis. This hypothesis states that a decrease in monoamines, such as serotonin or dopamine, in the synaptic cleft together with environmental factors, such as stress, can be an underlying cause of depression. Most antidepressant medications are based on this theory, and increase the amount of monoamines in the synaptic cleft. Unfortunately, antidepressant treatment alleviates symptoms of depression only after several weeks of medication in only a subset (~50%) of the patient

population (Berton and Nestler, 2006), indicating that there are also other mechanisms involved in the pathology of depression. To develop new therapeutic targets for depression, new concepts and hypothesis are needed described below.

The peptidergic hypothesis

Urocortin 1

One of such new hypothesis that focusses on the mechanisms underlying the stress response adaptation is the peptidergic hypothesis. This hypothesis postulates that if there is an imbalance in peptides involved in initiating and maintaining the stress response, it could lead to increased susceptibility to stress-related disorders. For over two decades, the HPA-axis, involving the neuropeptide CRF, has been considered the main system for controlling the stress adaptation. However, the identification of two CRF receptors (CRF-R1 and CRF-R2) with distinct ligand binding properties, added a new dimension to our view on stress adaptation. Moreover, the discovery of new members of the CRF neuropeptide family, urocortin 1 (Ucn1), urocortin 2 (or stresscopin-related peptide), and urocortin 3 (or stresscopin) has provided important insights into stress adaptation pathways and suggests that stress adaptation involves more systems than the HPA-axis alone (Steckler and Holsboer 1999, Bale and Vale 2004, Joëls and Baram 2009, Vaughan et al., 1995, Hsu and Hsueh, 2001, Lewis et al., 2001, Reyes et al., 2001, Janssen and Kozicz, 2013). Ucn1 is most abundantly expressed in the centrally projecting Edinger-Westphal nucleus (EWcp) (Kozicz et al., 1998; Bittencourt et al., 1999). It has been shown that Ucn 1 is involved in different behaviors such as the suppression of food and water intake (Spina et al. 1996, Jones et al. 1998, Smagin et al. 1998, Coste et al. 2000, Skelton et al. 2000), alcohol drinking (Ryabinin et al. 2012), social behavior (Sajdyk et al. 1999, Skelton et al. 2000) as well as in the stress response, depression, and anxiety (Moreau et al. 1997, Jones et al. 1998, Skelton et al. 2000, Gaszner et al. 2004, Kozicz 2007, Rotzinger et al. 2010, Kormos and Gaszner 2013). Furthermore, in humans it has been found that UCN1 mRNA is up-regulated in brain samples of male, but not female, suicide victims compared to non-depressed controls (Kozicz et al. 2008, Kormos and Gaszner 2013). In addition, these Ucn1 neurons are recruited by various acute stressors and their messenger RNA expression is up-regulated by acute pain and restraint stress (Kozicz et al., 2001; Cunha et al., 2007; Spencer et al., 2012). Our recent data and research by others indicate that an important role in stress adaptation is played by Ucn1 from the EWcp (Gaszner et al., 2004; Korosi et al., 2005; Cunha et al., 2007; Kozicz, 2007).

Orexin

Another family of peptides that seems involved in the stress response are the orexins, also known as the hypocretins, consisting of orexin-A and orexin-B. These peptides are synthesized solely within the lateral hypothalamus and adjacent regions (De Lecea et al., 1998; Sakurai et al., 1998). They bind to two G-protein-coupled receptors, orexin receptor-1 (OXR-1) and orexin receptor-2 (OXR-2) (Sakurai et al., 1998). Several observations suggest that the orexins modulate behavioral state and state-dependent processes. For example, narcolepsy is associated with a decreased concentration of orexin in the cerebrospinal fluid, as well as the number of orexigenic neurons is reduced (Peyron et al., 2000; Thannickal et al., 2000). Furthermore, intracerebroventricular administration of orexin-A or -B increases time spent awake as well as behaviors typical of spontaneous waking and/or stressful, high-arousal conditions, activate the HPA-axis, as well as activates CRF neurons in the paraventricular nucleus of the hypothalamus and the central nucleus of the amygdala (Kuru et al., 2000; Al-Barazanji et al., 2001; Samson and Taylor 2001; Sakamoto et al., 2004). Orexins are also involved in feeding behavior, the reward system, and the stress response (Sakurai et al., 1998; Kunii et al., 1999). Most of these functions are disturbed in depressed patients, indicating that orexin may be involved in depression. Indeed, several studies found a dysregulation of the orexinergic system in depressed patients (Nollet and Leman 2013, Chen et al. 2015). Also animal research supports the involvement of orexin in depression, different genetic animal models for depression (the Wistar-Kyoto and Flinders Sensitive Line rats) show differences in the orexin system compared to control animals (Allard et al. 2004, Mikrouli et al. 2011, Nollet and Leman 2013, Chen et al. 2015). However, how precisely orexins are involved in the pathology of depression is unknown, as in both human and animal research hypoactivity as well as hyperactivity of the orexin signaling is associated with depression and depressive phenotype (Nollet and Leman 2013, Chen et al. 2015). But with

aforementioned evidence it is reasonable to predict that the orexin system plays a role in depression pathology. In addition to this, various brainstem and basal forebrain regions that are implicated in the regulation of behavioral state of stress, including the locus coeruleus, the medial portion of the preoptic area, the paraventricular hypothalamic nucleus, and the EWcp, contain orexin-containing fibers and orexin receptors (Trivedi et al., 1998; Marcus et al., 2001; Peyron et al., 1998; Cutler et al., 1999; Date et al., 1999; Nambu et al., 1999). However, the exact mechanisms underlying the involvement of these peptides in behavioral and neuropsychological impairments that are observed in depression remain largely elusive.

MicroRNA hypothesis

As described above, dysregulated or altered peptide expression seems to play an important role in the adaptive stress response and depression susceptibility. In recent years a new player as regulator of peptide expression has emerged, namely microRNAs (miRNAs). MiRNAs are small, 21-nucleotide-long units of noncoding RNA which regulate gene expression post-transcriptionally. A miRNA first binds to a miRNA Silencing Complex (miRISC) after which this complex binds to a specific seed region on the 3' untranslated region of an mRNA. As the complex is bound to the mRNA, the mRNA is either degraded, translation is silenced, or in rare cases stimulated. One miRNA can bind up to hundred different mRNAs and, in addition, each mRNA can contain seed regions for hundreds of different miRNAs. To date, miRNAs have been linked to various processes such as metabolism (Ambros et al, 2008), cellular development, and apoptosis (Magni et al, 2014). Furthermore, different miRNAs are also linked to different diseases such as cancer (DeSano & Xu, 2009), Celiac disease (Magni et al, 2014), viral infections (Timoneda et al, 2014), neurodegenerative diseases (Cogswell et al., 2008; Maciotta et al, 2013), as well as psychiatric and stress related disorders (Kocerha et al, 2015). MiRNAs are potential important players in the development of stress related disorders because of their overall abundance in the brain, the potential regulation of different neuropeptides involved in the stress response, or because of their role in the regulation of various metabolic processes. Our preliminary findings on miR-326 show that this miRNA can directly regulate the Ucn1 peptide. Furthermore, others also have shown different miRNAs targeting e.g. CRFR1, glucocorticoid, and corticosteroid signaling (Kocerha et al, 2015, Haramati et al, 2011), all key mediators of the stress response as described above. Studies done on post-mortem brain tissue from depressed patients have shown changed expression levels of several miRNAs which might play a role in depression, including higher levels miR-1202 (Lopez et al, 2014) and lower levels of miR-135 (Issler et al, 2014). An ongoing, comprehensive study done by our lab on the expression patterns of noncoding RNA in the bed nucleus of the stria terminalis (BNST), amygdala, and prefrontal cortex (Broddman's area 25) of patients who suffered from depression and who eventually committed suicide, are showing several other interesting miRNAs including miR-34 and miR-127, which have been linked to stress-related anxiety (Haramati et al, 2011) and cocaine-induced plasticity (Chandrasekar & Deyer, 2011), respectively. As our experiment is ongoing, we will further functionally analyse these potential targets before we select several interesting ones for behavioural testing using the methods described in this project. This study should provide us with a list of microRNAs which are- and which aren't differentially expressed in highly stressed suicidal subjects as compared to a control condition. Whilst we are not sure yet which microRNAs will be tested, we will select those based on: a significant, consistent, differential expression in one or all of the brain areas involved, between the stressed- and non-stressed conditions ($P<0.05$; fold change of at least 1.2; preferentially a similar pattern of expression across brain areas); a similar gene expression of these microRNAs as measured by qPCR in human tissue of these stressed suicidal subjects; possibly similar differential protein expression as measured by luciferase vector assay, should we be able to select a strong regulatory target for the microRNA in question.

Suboptimal mitochondrial function hypothesis

All of the aforementioned processes require a substantial amount of energy mobilization, e.g. the production and regulation of peptides and synaptic plasticity required for a proper stress response. These processes are relative energy expensive processes. If the required amount of energy cannot be mobilized because of genetic defects or because the organism is exhausted, peptide expression and/or synaptic plasticity could be dysregulated. This in turn can lead to an incomplete or failed response to adapt to the stressor, leading to maladaptation. In the brain, most of the energy is

produced by the mitochondria. When mitochondrial function is not adequate for normal daily activities, it causes mitochondrial disorders. Patients with mitochondrial disorder show a 54% lifetime prevalence for depression (Fattal et al. 2007). This is more than two times as high as the incidence in the normal population which is around 20% (Kessler et al., 2003). Also, other studies showed that patients with suboptimal mitochondrial function had a higher incidence for developing depression compared to controls (Suomalainen et al., 1992; Carrozzo et al., 2007; Morava et al., 2006a, b; Koene et al., 2009). This indicates that decreased mitochondrial functioning could be pathological in depression. Furthermore, depressed patients show a decreased mitochondrial functioning in peripheral blood cells (Karabatsiakis et al, 2014). Similar to the monoamine hypothesis, a genetic predisposition in the functioning of the mitochondria together with a stressor may cause the individual to fail to adequately adapt to the stressor and develop stress related disorders such as major depression or anxiety (Morava and Kozicz, 2013). These findings, together with aforementioned required energy mobilization for an adequate stress adaptation, have led to the hypothesis that suboptimal mitochondrial function is involved in the pathology of depression. When not enough energy can be produced because of a decreased mitochondrial functioning, successful adaptation is not achieved and maladaptation may occur that ultimately can lead to stress related disorders such as depression or anxiety. Preliminary findings on mice with decreased mitochondrial functioning show that these animals are more prone to develop depressive behavior when stressed. Also immunohistochemical analysis shows an altered activation of several brain regions involved in the stress adaptation. In order to study this hypothesis a new animal model with decreased mitochondrial function will be used. This animal has a decreased Ndufs4 protein, a subunit of complex I of the mitochondria, because of a gene trap insertion in an early locus of the gene.

As described above, all three hypotheses have an influence on the stress response as well as on depression susceptibility. However, it seems that they also interact with each other. For example, miRNAs have a direct influence on different proteins such as Ucn1 (Aschrafi et al. 2015). Also the different described proteins such as Ucn1 (Lawrence et al. 2004, Townsend et al. 2007, Davidson et al. 2009) and orexin (Sellayah et al. 2011) have a direct influence on the mitochondria. A dysregulation of these proteins could also have a direct influence on mitochondrial functioning. In this way miRNAs can also have an indirect influence on the mitochondria by regulating or dysregulating different proteins. However, it has also been found that different miRNAs have a direct effect on the mitochondria (Chan et al. 2009, Chen et al. 2010). If this is also the other way around, that the mitochondria have a direct effect on the described proteins or miRNAs is unclear, this will also be investigated in this project. It seems that these three hypotheses are tightly linked, however what the mechanisms are, what processes are upstream or downstream, and what their influence is on depression symptoms are, is unknown. This project will contribute to unraveling this.

In this project we will also investigate the fact that depression is not only one disease. Depression has many different symptoms (e.g. Criteria A in DSM V) where only 5 out of 9 symptoms are required for the diagnosis depression. The different symptoms are depressed mood or irritable, decreased interests or pleasure, significant weight change or change in appetite, change in sleep, change in activity, fatigue or loss of energy, guilt/worthlessness, concentration, and suicidality. The different animal models will not only be used because they have interacting mechanisms, but also because they can shed light on potential different mechanisms underlying different symptoms of depression. Because only 5 out of 9 symptoms must be present to diagnose depression, there are many different combinations possible resulting in the fact that not everyone have the same symptoms. This can point towards different mechanisms that we can potentially investigate with the different animal models. For example, this way we can investigate if orexin influences the change in weight, appetite or sleep as depression symptoms, or that the mitochondria are underlying the fatigue, loss of energy or a change of activity. Together with the three different stress models we try to elucidate the potential different underlying causes of depression that can explain the different symptoms with different people.

So, despite several decades of research the current knowledge and therapies for the treatment of depression are not yet sufficient. This is largely because the exact underlying mechanisms of depression are still largely unknown. In this project we will test several new hypothesis for underlying

causes of depression, namely the involvement of the peptides orexin and urocortin as well as microRNAs and suboptimal mitochondrial functioning. This will be done using readily available animal models, namely the orexin KO, Ucn1 KO, and Ndfs4 deficient animals, as well as with mice where a specific miRNA expression is altered. This project also utilizes three different kinds of stressors, namely chronic variable stress, chronic social defeat stress, and acute stress. These three stressors are necessary because it is known that physical and psychological stressor have other effects on different individuals and on underlying mechanisms (Lamb 1979, Briski and Gillen 2001, Kavushansky et al. 2009). The chronic variable stress focusses more on the physical and unpredictable component of the stressor while the social defeat stress focusses more on the psychological component of stress. To determine if the initial stress response is different between WTs and the different animal models in this project an acute stressor is used.

Lastly, an intervention study is utilized to test the influence of different antidepressants on depressive behavior in the stressor that gives the most robust effect in each animal model. This experiment will be done using two different classical antidepressants. These antidepressants will be chosen on for example their effect on the mitochondria. It is known that different antidepressants have a different effect on the mitochondria, some will have a positive effect whilst others have a negative effect on mitochondrial function. This intervention experiment will verify and potentially strengthen results found in first three stress experiments. Classical antidepressants are used because they are readily available and widely used, consequently if this research finds promising new results and uses for these classical antidepressants this could faster lead to more personalized health care.

With this project we aim to shed more light on potential underlying causes and mechanisms for depression. Hopefully with the acquired new insight in the aethiology of depression new and more effective treatments can be generated.

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?

This project aims to provide several important novel insights in the etiology and biological underpinnings of stress related disorders such as depression using various translational animal models. For example, the involvement of mitochondria in the susceptibility in depression, the involvement of certain microRNAs in the pathophysiology of stress related disorders, as well as the involvement of different peptides such as Ucn1, orexin, and CRF in the stress response and depression susceptibility. In addition to this, possible interactions between these different biological underpinnings will also be investigated as well as how much these could contribute to the pathology of various Category A depression symptoms (DSM V) like fatigue, sleep problems, psychomotor retardation, etc.. The use of novel hypotheses, as outlined in section 3.1, in combination with well-validated models for stress-related psychological diseases with the focus on depression, will help us better understand the underlying mechanisms of stress related psychiatric diseases as well as a better understanding of the various category A symptoms of depression. Consequently, it could also give new insights in identifying novel therapeutic targets and treatment strategies of major depression. In addition, important data will be acquired to better understand fundamental mechanisms contributing to stress adaptation, and consequently will increase our insight into neuronal processes that may underlie unsuccessful adaptation to stress and depression susceptibility. Ultimately, this may lead to the development of new treatment targets or the re-categorization of current treatments which is investigated in experiment 4. In order to investigate the interaction between the above detailed mechanisms in depression vulnerability, the various mechanisms mentioned in the background are

examined in parallel. We will be able to identify different behavioral, physiological, and endocrine aspects of depression that can be correlated/compared to similar parameters in humans giving this project a great translational value.

Similar experiments investigating stress-related disorders were already performed in the past in our lab including experiments with Ndufs4def, orexin KO, and Ucn1 KO mice, giving us experience with these types of experiments. This allows us to work efficiently and with the least amount of distress for the animals. Also, because of this experience and the available knowledge, the main objectives should be achievable and realistic within the duration of the project. This project will be a continuation of the research that has already been performed in this lab towards unraveling the underlying mechanisms of depression. Previous experiments encompass miRNA 326 and Ucn1 (Aschrafi et al. 2015), orexin and stress (Emmerzaal et al. 2013), Ucn1 and depression (Kozicz 2007, Spencer et al. 2012), as well as mitochondrial function and depression (Emmerzaal et al. 2015).

3.3 Relevance

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

It has been estimated that one in five individuals will develop depression in some point in life and in 2010 there were 298 million separate cases of depression recorded globally, with an average duration of 37.7 weeks (Ferrari et al, 2013). At that time depression was the second leading cause of burden of disease worldwide (Ferrari et al, 2013), whilst the World Health Organization (WHO) estimated that depression would be the leading cause of disease by the year 2020. At this moment the WHO reports that depression is the leading cause for disease with worldwide 350 milion people suffering from depression of all ages. Depression not only has a profound effect on the individual but also on his/her family and society as a whole. Despite decades of research towards the pathogenic mechanisms behind depression, the neurobiology underlying this complex disorder remains largely elusive. A consequence is that treatment options at the moment remain poor, with up to 40% of patients not responding to current treatment methods (Fava, et al, 1996; Berton and Nestler, 2006; Saad Al-Harbi, 2012). Therefore, understanding the (neuronal) mechanisms underlying fundamental biological processes in adaptation to stressors are of great importance (as the link between stress adaptation and depression indicated in section 3.1). Despite several decades of research that have identified several possible underlying pathologies, progress in understanding depression, and related disorders overall, has been slow and the search for new therapeutic targets and approaches is necessary. In this context, fundamental animal research utilizing well-validated animal models of depression is therefore of great importance to reveal novel mechanisms of stress-related psychiatric diseases that can lead to novel treatment strategies.

3.4 Research Strategy

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

The main objective of this project is to investigate underlying mechanisms that mediate the behavioral, endocrine and physiological alterations seen in depression, a stress-related disorder. In these experiments the different genes of interest and possible underlying mechanisms described in section 3.1 will be investigated using different animal models. These animals are bred for a specific genetic knockout/knockdown and therefore have a general change in expression of the gene of interest. In these experiments we will be using mice which lack orexin and Ucn1. We will also use an Ndufs4 deficient mouse model to induce a decreased mitochondrial function in these animals. These animals are readily available in our lab. Furthermore, also two different miRNAs will be tested through either lenti-/adeno-viral injections or through specific ‘floxing’ of animals and

employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The specific miRNA will be chosen after a comprehensive investigation in suicide patients that is ongoing at this moment as described in section 3.1. Using these genetically modified animals, we will aim to investigate whether changes in the expression of these genes or miRNAs, coupled with different methods of stress induction influence depression susceptibility. To investigate depression susceptibility three different stress paradigms will be used, 1) a chronic variable stress paradigm, 2) chronic social defeat stress paradigm, and 3) an acute stress paradigm. 4) After these three experiments an intervention study with different antidepressants will be utilized with the stress model that gives the most robust findings in the animals.

The five different animal models (Ucn1 KO, orexin KO, Ndufs4def, altered miRNA mice) will be used in four different experiments. All these animal models will be used to study the following:
1) Investigate the effect of unpredictable chronic stress on depression susceptibility.
For this experiment the different animal models are subjected to a chronic variable stress paradigm and their depression susceptibility is determined via various behavioral tests and biochemical parameters.
The questions to be answered in this experiment are:
- Is depression susceptibility after a chronic variable stress paradigm influenced by altered peptide levels, mitochondrial function, or miRNA abundance?
- What are the specific effects of these altered parameters on the animals behavior after the stressor?
- What biochemical parameters underlie and influence the potential depression susceptibility?

2) Investigate the effect of social defeat stress on depression susceptibility.
For this experiment the different animal models are subjected to social defeat stress. This will be induced via a resident intruder paradigm. Depression susceptibility is again determined via various behavioral tests and biochemical parameters.
The questions to be answered in this experiment are:
- Is depression susceptibility influenced by altered peptide levels, mitochondrial function, or miRNA abundance after social stress?
- Are other mechanisms involved in depression susceptibility after social stress as compared to chronic variable stress?

3) Investigating differences during the acute stress response as possible underlying causes for depression susceptibility.
In this experiment the different animal models will be challenged by an acute stressor to determine if the initial stress response is already altered in these animals.
This experiment addresses the following questions:
- Can the potential differences found during the chronic stressors be explained by a difference during the acute stress response?
- Are different acute stress parameters negatively altered in the different animal models?
- Is there a difference in neuronal network activation during the initial stress response?

4) The effect of different antidepressants on reducing depression susceptibility
In this experiment one of the three stressors will be used in an intervention experiment. The animals will be stressed while different groups of animals will receive different treatments. These different treatments are for example based on the effect at the mitochondria.
With this experiment we can address the following questions:
- Can the enhancement or decrease of mitochondrial function by antidepressants influence depression susceptibility?
- Are current antidepressants effective in the treatment of depressive behavior in these different animal models?

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.

First we plan to investigate the effects of different stressors on depression susceptibility in the different animal models as explained in the previous sections. We will use mice which lack the peptides Ucn1 and Orexin, as well as a new mouse model with decreased mitochondrial function because of a lower Ndufs4 expression. These animals are readily available and present in our lab. To investigate the influence of miRNA abundance we will use specifically engineered viral vectors that can be injected locally, to induce changes in microRNA abundance. To investigate depression susceptibility of these genetically modified animals they will be subjected to three different well validated stress paradigms; 1) chronic variable stress (Pittenger and Duman 2008, Hill et al. 2012, Franceschelli et al. 2014), 2) chronic social defeat stress through a resident intruder paradigm (Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991, Rygula et al. 2005), and 3) acute stress (Katz et al. 1981, Fullerton et al. 2004, Bogdan and Pizzagalli 2006).

1) The chronic variable stress model is based on the learned helplessness aspect of stress. The animal is not able to control or predict the stress it will face, due to its unpredictable– and chronic nature. The chronic stress will consist of a variable stress paradigm where for 21 consecutive days a stressor and/or a behavioral test will be presented to the animals. These stressors will be presented in a randomized order where each stressor is presented an equal number of times. The duration of the stressor can be between one hour and an overnight time period. All animals in the stressed group will receive the same stressors.

2) The social defeat stress model is based on stress originating from the experience of aggression and submission. This will be done via a social defeat paradigm. Previous research has shown social defeat stress to be a viable animal model for depression, that induces a depressive phenotype (Kudryavtseva, Bakshtanovskaya, & Koryakina, 1991; Rygula et al., 2005) and an enduring activation of the HPA axis (Covington & Miczek, 2005; Koolhaas, De Boer, De Rutter, Meerlo, & Sgoifo, 1997).

3) Acute stress will be used to study changes in the initial phase of the stress response that could eventually lead to a depressive phenotype. The stressor can be for example a series of electric foot shocks, restraint, lipopolysaccharide (LPS) injection, or forced swimming.

After each of the aforementioned stress procedures several well validated behavioral tests will be performed in order to investigate normal- and depression-like behavior. Behavioral tests that will be used are for example the Porsolt swim test, sucrose preference test, novelty suppressed feeding, open field test, Rotarod, and grip test. In addition to these tests, the animals will be implanted with a telemetry sensor that can measure autonomous functions such heart rate and core body temperature. This gives information about the rhythm and possible autonomous disturbances the animals may have or develop during the stress paradigm. In order to measure brain structure and function in vivo, neuroimaging techniques are used, such as Diffusion Tensor Imaging (DTI), resting state functional MRI (rs-fMRI) and Magnetic Resonance Spectroscopy (MRS). Shortly after the last stressor- and behavior experiments the animals will be sacrificed and biological material will be collected for further histological and biochemical analyses.

4) Lastly, after these three experiments, an intervention study will be done utilising the stress model that gives the most robust findings in the animals. This can differ for the different genetically modified animals. The intervention will encompass two different classical antidepressants. Different antidepressants will be used because it is for example known that some antidepressants have a positive while others have a negative effect on the mitochondria, possibly influencing depression susceptibility for better or for worse (Klinedinst and Regenold 2015). The choice of the specific

antidepressants will be made after all three stress experiments are executed. At this moment this choice cannot be made because it depends on the obtained results as well as the advances that are being made for different effects, and potentially newly discovered antidepressants.

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 individual components of this project focus on understanding the influence of the different described peptides, mitochondrial function, and miRNAs on depression susceptibility after stress and the potential interactions between these different mechanisms.

The four proposed animal procedures will be executed in succession with each other (see figure 1). This flowchart will be used for each of the five proposed animal models (orexin KO, Ucn1 KO, Ndufs4 deficient, and two virally injected groups). Between the different animal procedures there will not be a go/no-go evaluation moment.

Each animal procedure is first executed with behavioral testing and biochemical and histological analysis only. After the experiment is completed, there is a go/no-go evaluation moment, if differences are found between WT and genetically modified animals MRI experiments will also be utilized (Figure 1). The same paradigm with behavioral tests is executed before the MRI. If no differences are found no MRI experiment will be implemented (see figure 1) and the next animal procedure can be executed. The MRI protocol includes DTI, rs-fMRI, and MRS to investigate functional and structural differences between animals in vivo.

Before the start of animal procedure 4, there is another go/no-go evaluation moment. All three animal procedures are evaluated and compared for that specific animal model. The paradigm that gave the most reliable and robust results will be used for the intervention experiment to minimize the number of animals used. **Also, if less than three behavioral tests are interesting to investigate in this experiment we will use only one cluster of behavioral tests and not two clusters as in the previous experiments, thus reducing the number of animals used.**

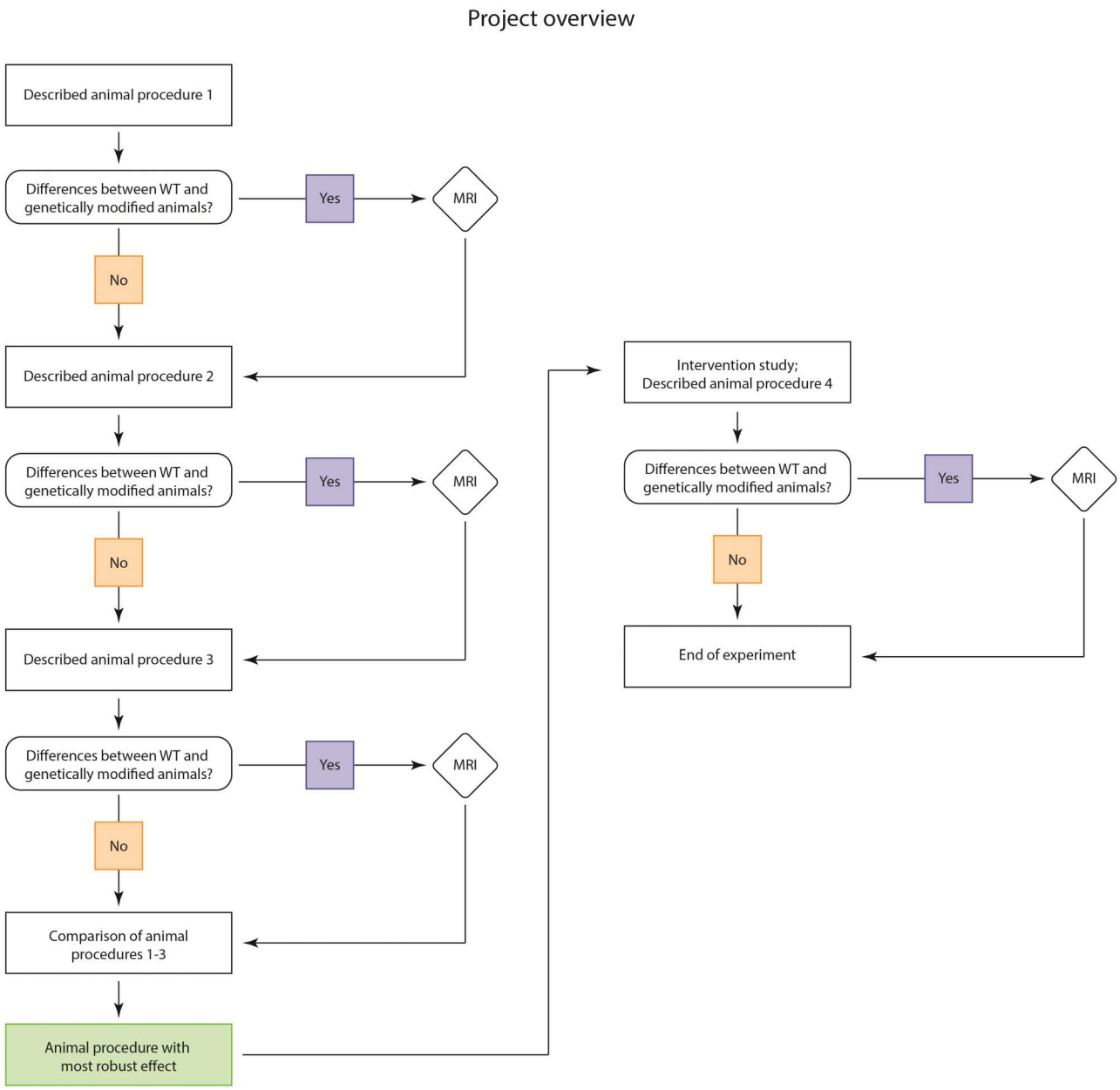

Figure 1: Project overview

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	Chronic variable stress paradigm
2	Chronic Social defeat stress
3	Acute stress paradigm
4	Intervention experiment

10.

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	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 Chronic variable stress paradigm

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.

Different animal models will be used in order to study whether altered protein expression, and/or decreased mitochondrial function can affect depression susceptibility. The protein expression is either constitutively altered (orexin KO and Ucn1 KO mice) or can be influenced with miRNAs. Orexin KO and Ucn1 KO mice are readily available and used in this project because these proteins have been shown to be involved in depression susceptibility as described in section 3.1 of the project proposal and also to continue previous research in our lab. Urocortins 2 and 3 will not be used in this project because although they are paralogous to each other and are part of the CRF family, they are distinct from CRF and urocortin 1 (Rotzinger et al. 2010). The specific miRNAs of interest will be determined after the comprehensive study mentioned in section 3.1 of the project proposal is finished, as of now miR-34 and miR-127 seem promising targets. When the miRNAs of interest are determined they will be influenced either through lenti-/adeno-viral injections or through specific 'floxing' of animals and employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The actual choice will depend on the gene/microRNA in question, and whether there is a 'floxed' animal model available. To investigate the influence of decreased mitochondrial functioning on depression susceptibility a new mouse model with a gene trap insertion in the Ndufs4 allele will be used. This genetic alteration results in a premature termination of the coding sequence and deficiency of the NDUFS4 protein. This deficiency results in a decreased mitochondrial complex I and III activity of the electron transport chain, but the animals behavior remains normal under basal conditions.

The different above described animal models will be subjected to a chronic variable stress paradigm, a well validated and often used paradigm to induce experimental depressive-behavior in animals. We will assign both the genetically modified animals as well as WT mice randomly to the chronic stress or control group. Control mice will be exposed to similar conditions compared to mice in the chronic variable stress group, but without exposing them to the stressor. During and after the chronic stress paradigm mice will be subjected to several behavioral tests to monitor the effect of the chronic stress on the animal's behavior. Different behavioral tests will measure depressive behavior (e.g. novelty suppressed feeding, forced swim test, tail suspension test) as well as well as general behavior such as locomotor activity (e.g. RotaRod, Phenotyper, open field). The weight of the animal will also be measured several times during the paradigm. Shortly after the last stressor or behavioral test the animals will be sacrificed, blood and biological material will be collected, such as the brain, and several other organs. If robust changes are found, these studies are followed up by magnetic resonance imaging (MRI) to study brain structure and function, including DTI, rs-fMRI, and MRS. The neuroimaging will be executed on a different group of animals because it is known that isoflurane, that is used to anesthetize the animals, has an influence on mitochondrial function and on the stress response parameters. These animals will also undergo the different behavioral tests.

In order to determine the effect of the stressor on autonomic functions in the different animal models such as body temperature, as well as overall activity and sleep patterns, a telemetry sensor that can measure these will be implemented in the mice. This will both be in WT as well as genetically modified animals. The sensor will be implemented in these animals minimal two weeks before the stress procedure in order for the animals to recover from surgery. In this time period it will also be possible to set a baseline for each animals before the stress experiment.

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or mRNA in the brain, and mitochondrial function. These primary readout parameters will be used because it is known for example body weight decreases, plasma corticosterone increases, and mitochondrial function is altered during the stress paradigm. Furthermore these read out parameters are well-validated markers for depressive behavior, widely used, and easily measured. If a robust statistically significant difference between the different groups is found, neuroimaging will also be conducted. In order to correlate biochemical, behavioral and neuroimaging data to each other the animals that undergo neuroimaging will also be behaviorally tested.

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

Our aim is to compare genetically modified- or virally injected animals to WT littermates (non-genetically modified or control virally injected (injected with a virus containing a scrambled target sequence) respectively), by exposing them to a chronic stress paradigm. During and after the stress paradigm the animals will be exposed to several standard behavioral tests devised to measure depression-related behavior. Mice will randomly be assigned to the either the control group or the stress group.

The telemetry sensor that will be implanted can measure core body temperature and activity. Animals will be operated minimally two weeks prior to the stress induction to allow enough time for the animals to recover. The telemetry sensor will be placed subcutaneaus under general anesthesia (1.5 – 2% isoflurane inhalation).

The miRNA abundance will be influenced via local viral injections in the brain. The virus will be injected during the same operation as the telemetry sensor implantation to allow enough time for incubation and also recovery from surgery and to reduce discomfort of two separate surgeries. The viral injection is done while the animal is placed in a restraint / stereotactic apparatus, whilst the viral construct can be delivered through a thin syringe and needle.

The genetically modified animals, as well as the WT littermates, are randomly assigned to either the experimental- or the control condition. Both groups will follow similar protocols, with the only difference that the experimental group will be stressed, and the control group will not receive stress. In this experiment, chronic stress is induced by a chronic variable stress paradigm. This paradigm consists of 21 successive days where each day a stressor and/or a behavioral test is presented to the animal. Each animal in the stress group receives the same stressors and these stressors will be presented in a randomized order where each stressor, mentioned in table 1, is presented an equal number of times (except for the behavioral tests mentioned in the table). The duration of the stressor varies between one hour and an overnight time period, depending on the stressor. The stressors that will be used are for example restraint stress, cold stress, confinement, continuous light overnight, social isolation overnight, soiled cage overnight, shaking stress, cage tilting, overnight food deprivation (for more details see table 1). This paradigm is based upon previously experiments as well as described regimens and is well validated and a proven mouse model for depressive behavior in rodents (Stout et al. 2000, Willner 2005, Deussing 2007, Franceschelli et al. 2014).

Table 1. Different stressors used during the chronic variable stress paradigm. These stressors will be presented to the animals in a randomized order one a day.

Stressor	Average duration	Short description	Discomfort level
Confinement	Repeated 1h periods	Animals are placed in a small cage	Moderate
Cold stress	1 hour	Animals are exposed to 4°C	Severe
Continuous light	Overnight	Animals are exposed to continuous light overnight	Moderate
Social isolation	Overnight	Animals are isolated overnight; one mouse/cage. This can be for example combined with an Phenotyper for behavioral analysis	Mild
Soiled cage	Overnight	Bedding material will be soiled with water (±250 ml)	Severe
Shaking stress	1 hour	Cages are placed on an orbital shaker (±100 rpm)	Moderate
Restraint	30 min	Animals are placed in a plastic restrainer	Moderate
Cage tilting	Overnight	The home cage of the animals will be tilted overnight	Moderate
Food deprivation	Overnight	Animals will not have access to ad libitum food overnight	Moderate
Novelty suppressed feeding	10 min	Animals will be placed in a novel environment where a food pallet is placed in the middle; executed after overnight deprivation	Mild
Porsolt swim test	10 min	Animals are placed in a cylinder filled with water and are forced to swim for 6 minutes	Severe
Tail suspension	10-15 min	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces.	Moderate

At the end of the stress paradigm general and depressive related behavior of the animals will be analyzed with several different behavioral experiments. For example the Porsolt swim test, tail suspension test, sucrose preference test, novelty suppressed feeing, open field test, Rotarod, splash test, and grip test. The behavioral tests that have a higher discomfort for the animals, Porsolt swim test and tail suspension test, will not be executed on the same animal. These two tests will be executed with two other milder behavioral tests. Short descriptions of these tests are included in table 2. Each behavioral test is executed once at the end of the stress paradigm.

Table 2. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

On the 21st day, shortly after the lasts behavioral test, the animals will be sacrificed and blood will be taken. The sacrificing method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood, and several other organs will also be collected for additional analyses.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size was based and calculated using standard deviations from the behavioral test with the most variation (FST) from previous experiments. The effect size was $d=0.982$, giving a minimal number of animals of 14 per group. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 14 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly besides the stress paradigm. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the experimental- or control condition. This will be to strengthen the study design by limiting variation between groups due to experimenter buis and housing influences.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the new Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

Previous studies and experience in our lab using comparable chronic variable stress-paradigms have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. We need a minimum of 14 animals per group, however, we do not want to expose each animal to more than 3 behavioral tests post-stressor/non-stressor and we aim to do 4 to 6 behavioral tests. Therefore another group of animals has to be added for the additional behavioral tests, totaling at 28 animals per group (see table 3). The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of 14 animals per group, for details see table 3.

Table 3. Maximal number of animals necessary

Animal model	Number of animals per group				Total
	No-MRI		MRI		
	Control	Stress	Control	Stress	
Orexin KO / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
Ucn1 KO / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
Ndufs4def / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
MiRNA 1 / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
MiRNA 2 / WT	28 / 28	28 / 28	14 / 14	14 / 14	180
Total	140 / 140	140 / 140	70 / 70	70 / 70	900

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	900	Adult

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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The model we chose is one of the best validated animal models for stress-related psychopathology, and although it uses a chronic stress exposure, it results in relative mild/moderate physical and psychological distress/harm compared some other stress-paradigms. The behavioral tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioral changes. Viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anesthesia (via 1.5% isoflurane inhalation) in order to minimize animal suffering. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that chronic stress causes discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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.

Non-applicable

Accommodation and care

F. Accommodation and care

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

[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?

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

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

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

During viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of 1.5% isoflurane inhalation. Otherwise no anesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

During the chronic stress paradigm we will not apply anesthesia or analgesia. When mitochondrial function will not be tested, and when perfusion will be used as a means of sacrifice, animals are first anesthetized by means of 1.5% isoflurane inhalation.

I. Other aspects compromising the welfare of the animals

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

Stress will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, a chronic stressor has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to chronic stress, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful will be implemented in the chronic stress paradigm to monitor their behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The chronic variable stress paradigm is a widely used approach to investigate stress adaptation and stress associated disorders such as depressive behavior. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behavior (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the chronic stress paradigm or have complications from surgery. Animals will constantly be monitored for signs of sickness, infection, excessive weight loss, or other signs of diminished well being. If an animal during the

experiment shows a body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or veterinarian. Because of the non-invasive nature of the stressors and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The stressor is of a non-invasive nature whereby the incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments. Based upon our previous experience with similar experiments with the Ucn1 KO mice, we expect to have less than 3% of the animals develop these criteria.

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 the animals experience at the end of the chronic stress paradigm and behavioral tests is considered severe. The effect of the behavioral tests is expected to be mild to severe, depending on the behavioral test. The cumulative discomfort of the animals subjected to chronic stress is severe; for control mice this cumulative discomfort will moderate or severe, as is classified is Annex VIII of the Directive 2010/63/EU. The discomfort severity of the control mice depends on the cluster of behavioral tests, if the cluster includes the Porsolt swim test it will be severe (50% of the control animals), if not and it includes the tail suspension test it will be moderate (50% of the animals). In total, 75% of the animals will experience severe discomfort and 25% of the animals will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without

these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 2	Type of animal procedure Chronic Social defeat stress

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.

Different animal models will be used in order to study whether altered protein expression, and/or decreased mitochondrial function can affect depression susceptibility. The protein expression is either constitutively altered (orexin KO and Ucn1 KO mice) or can be influenced with miRNAs. Orexin KO and Ucn1 KO mice are readily available and used in this project because these proteins have been shown to be involved in depression susceptibility as described in section 3.1 of the project proposal and also to continue previous research in our lab. Urocortins 2 and 3 will not be used in this project because although they are paralogous to each other and are part of the CRF family, they are distinct from CRF and urocortin 1 (Rotzinger et al. 2010). The specific miRNAs of interest will be determined after the comprehensive study mentioned in section 3.1 of the project proposal is finished, as of now miR-34 and miR-127 seem promising targets. When the miRNAs of interest are determined they will be influenced either through lenti-/adeno-viral injections or through specific 'floxing' of animals and employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The actual choice will depend on the gene/microRNA in question, and whether there is a 'floxed' animal model available. To investigate the influence of decreased mitochondrial functioning on depression susceptibility a new mouse model with a gene trap insertion in the Ndufs4 allele will be used. This genetic alteration results in a premature termination of the coding sequence and deficiency of the NDUFS4 protein. This deficiency results in a decreased mitochondrial complex I and III activity of the electron transport chain, but the animals behavior remains normal under basal conditions.

The different animal models will be subjected to a chronic social stressor by using the well-validated Resident-Intruder paradigm, which uses repeated aggressive confrontations to induce social defeat. The animals will be subjected to a 10-day period of daily confrontations, followed by a social dominance test. Previous research has shown social defeat stress to be a good animal model for depression, whereby not only depressive behavior is induced (Kudryavtseva, et al., 1991; Rygula et al., 2005), but also a robust enduring activation of the HPA axis (Covington & Miczek, 2005; J M Koolhaas er al., 1997). We will assign both the genetically modified animals as well as WT mice randomly to the social defeat stress or control group. Control mice will be exposed to similar conditions compared to mice in the chronic stress groups, but these mice will be introduced to a non-aggressive mouse strain. To prevent the stress of confrontation, the cage will be fitted with a barrier to allow the animals to smell, see, and hear each other, however which will prevent the actual interaction. After the stress paradigm mice will be subjected to several behavioral tests to monitor the effect of the stress on the animal's behavior.

If robust changes are found, these studies are followed up by magnetic resonance imaging (MRI) to study brain structure and function, including DTI, rs-fMRI, and MRS. The neuroimaging will be executed on a different group of animals because it is known that isoflurane, that is used to anesthetize the animals, has an influence on mitochondrial function and on the stress response parameters. These animals will also undergo the different behavioral tests.

Different behavioral tests will focus on depressive behavior in these animals. Also general behavior such as locomotor activity will be tested (e.g. RotaRod, Phenotyper, and Open Field). The weight of the animal will also be measured several times during the paradigm. Shortly after the last behavioral test the animals will be sacrificed, blood and biological material will be collected, such as the brain, and several other organs. In order to determine the effect of the stressor on autonomic functions in the different animal models such as body temperature, as well as overall activity and sleep patterns, a telemetry sensor that can measure these will be implemented. This will both be WT as well as genetically modified animals. The sensor will be implemented in these animals minimal two weeks before the stress procedure in order for the animals to recover from surgery. In this time period it will also be possible to set a baseline for each animals before the stress experiment.

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or mRNA in the brain, and mitochondrial function. These primary readout parameters will be used because it is known for example body weight decreases, plasma corticosterone increases, and mitochondrial function is altered during the stress paradigm. Furthermore these read out parameters are well-validated markers for depressive behavior, widely used, and easily measured. If a robust statistically significant difference between the different groups is found, neuroimaging will also be conducted. In order to correlate biochemical, behavioral and neuroimaging data to each other the animals that undergo neuroimaging will also be behaviorally tested.

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

Our aim is to compare genetically modified- or virally injected animals to WT littermates (non-genetically modified or control virally injected (injected with a virus containing a scrambled target sequence) respectively), by exposing them to a chronic social defeat paradigm followed by several standard behavioral tests devised to measure depression-related behavior. Mice will randomly be assigned to the either the control group or the stress group.

The telemetry sensor that will be implanted can measure core body temperature and activity of the mice. Animals will be operated minimally two weeks prior to the stress induction to allow enough time for the animals to recover. The telemetry sensor will be placed subcutaneous under general anesthesia (1.5 – 2% isoflurane inhalation).

The miRNA abundance will be influenced via local viral injections in the brain. The virus will be injected during the same operation as the telemetry sensor implantation to allow enough time for incubation and also recovery from surgery and to reduce discomfort of two separate surgeries. The viral injection is done while the animal is placed in a restraint / stereotactic apparatus, whilst the viral construct can be delivered through a thin syringe and needle.

The genetically modified animals, as well as the healthy WT littermates, are randomly assigned to either the experimental- or the control condition. Both groups will follow similar protocols, with the only difference being that the experimental group will be introduced to very aggressive BALB/cJ mice while the control animals will be introduced to C57BL/6J mice. The control mice will be in the same cage as the resident and while the mice are being able to see, hear, and smell each other they are not able to have physical contact. Over a ten day period the animals will be introduced daily to

a different resident. Our particular confrontation protocol is similar to DEC-nr.: 2013-235, and the protocol by Koolhaas et al (2013). Animals will be in each others' presence for no longer than 10 minutes (attack latency), in which the attacks can take place. An session will last no longer than 5 minutes after the first attack.

At the end of the stress paradigm general and depressive related behavior of the animals will be analyzed with several different behavioral experiments. For example the Porsolt swim test, tail suspension test, sucrose preference test, novelty suppressed feeing, open field test, Rotarod, splash test, and grip test. The behavioral tests that have a higher discomfort for the animals, Porsolt swim test and tail suspension test, will not be executed on the same animal. These two tests will be executed with two other milder behavioral tests. Short descriptions of these tests are included in table 4. Each behavioral test is executed once at the end of the stress paradigm.

Table 4. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

Afterwards blood samples will be acquired and the animals will be sacrificed whereby the method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood plasma, and several other organs will also be collected for additional analysis.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size was based and calculated using standard deviations from the test with the most variation from previous experiments. The effect size was $d=1.05$, giving a minimal number of animals of 12 per group. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 12 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly besides the social stress paradigm. During the social stress paradigm residents/intruders will be mixed constantly in a way that residents and intruders will never be confronted twice with each other to avoid recognition. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the experimental- or control condition. These considerations will be to strengthen the study design by limiting variation between groups due to experimenter bias and housing influences.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

To induce chronic stress through a social defeat paradigm two main groups of animals are needed: a group of residents and a group of intruders (the animals of interest, including the below described genetically modified animals). The residents are required to be more aggressive and dominant that the intruders, in order to induce a stable level of stress and social defeat. BALB/cJ mice will be used as residents, besides being slightly bigger compared to the C57BL/6J mice, they are also more aggressive (Fairless et al., 2012, Velez, et al., 2010). Besides the aggressive BALB/cJ mice, we will use WT littermates as non-aggressive normal behaving animals as a control condition.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

Previous studies and experience in our lab using comparable chronic social defeat stress-paradigms have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. We need a minimum of 12 animals per group, however, we do not want to expose each animal to more than 3 behavioral tests post-stressor/non-stressor and we aim to do 4 to 6 behavioral tests. Therefore another group of animals has to be added for the additional behavioral tests, totaling at 24 animals per group (see table 5). The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per

animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

The number of residents required for this experiment is half of the number of intruders described above (see table 5). Only half of the animals is necessary because we expect that we can use the resident mice in two separate experiments. However, we will ensure that each intrudes see a different resident every time to avoid recognition between the animals.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of 12 animals per group, for details see table 5.

Table 5. Maximal number of animals necessary

Animal model	Number of animals per group				Total
	No-MRI		MRI		
	Control	Stress	Control	Stress	
Orexin KO / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
Ucn1 KO / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
Ndufs4def / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
MiRNA 1 / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
MiRNA 2 / WT	24 / 24	24 / 24	12 / 12	12 / 12	144
Total	120 / 120	120 / 120	60 / 60	60 / 60	720

	WT	BALB/cJ	WT	BALB/cJ	
Residents	120	120	60	60	360
Total:	360	360	180	180	1080

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	1080	Adult

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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The model we chose is one of the best validated animal models for stress-related psychopathology, and although it uses a chronic stress exposure, it results in relative mild/moderate physical and psychological distress/harm compared some other stress-paradigms. The behavioral tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioral changes. Viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anesthesia (via 1.5% isoflurane inhalation) in order to minimize animal suffering. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that chronic social defeat stress causes discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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.

E. Repetition

Non applicable

Accommodation and care

F. Accommodation and care

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

[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?

H. Pain and pain relief

[] No > Continue with question I.

[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

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

During viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of 1.5% isoflurane inhalation. Otherwise no anesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

During the social defeat paradigm we will not preform anesthesia or analgesia. When mitochondrial function will not be tested, and when perfusion will be used as a means of sacrifice, animals are first anesthetized by means of 1.5% isoflurane inhalation.

I. Other aspects compromising the welfare of the animals

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

Stress and single housing of the residents will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, a chronic stressor has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. . During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anaesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to social defeat stress, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful are executed after the resident intruder paradigm to determine their behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The stressors' nature is non-invasive, and duration will be strictly monitored, in order to minimize physical- and mental distress, whilst still being able to induce differences between treatment groups. We have selected a maximum time of each separate confrontation (10 minutes), with a maximum of 5 minutes after the first attack. Also, we will make sure that no excessive physical harm is done; we will separate animals and terminate the confrontation prematurely if we deem the risk of excessive physical harm too high. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behaviour (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the chronic stressor or have complications from surgery. Animals will constantly be monitored for signs of sickness, infection, large open wounds, other extreme physical harm, excessive weight loss, or other signs of diminished wellbeing. If an animal during the experiment shows a body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or veternarian. Because of the non-invasive nature of the stressor and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The stressor is of a non-invasive nature, whereby we will closely monitor the attacks, perform proper wound care, and make sure the animals eat well. The incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments. We expect to have less than 5% of the animals develop these criteria.

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 the animals experience from the intruder's point of view in the resident intruder paradigm is considered moderate to severe, depending on the resident they will face. The effect of the behavioral tests is expected to be mild or severe, depending on the behavioral test. The cumulative discomfort of the animals subjected to the aggressive Balb/cJ mice (50%) in combination with the behavioral tests is severe; for the intruders which will face the C57BL/6J mice this cumulative discomfort will be moderate to severe depending on the behavioral tests used. The discomfort for the residents will be moderate, as is classified is Annex VIII of the Directive 2010/63/EU.
The mice that will face the C57BL/6J mice and receive the Porsolt swim test will have severe discomfort (50%) while the animals that face the C57BL/6J mice and do not receive the Porsolt swim test will have moderate discomfort (50%). In total, 37.5% of the animals (including both residents and intruders) will experience severe discomfort and 62.5% of the animals will experience moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 3	Type of animal procedure Acute stress paradigm

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.

Different animal models will be used in order to study whether altered protein expression, and/or decreased mitochondrial function can affect depression susceptibility. The protein expression is either constitutively altered (orexin KO and Ucn1 KO mice) or can be influenced with miRNAs. Orexin KO and Ucn1 KO mice are readily available and used in this project because these proteins have been shown to be involved in depression susceptibility as described in section 3.1 of the project proposal and also to continue previous research in our lab. Urocortins 2 and 3 will not be used in this project because although they are paralogous to each other and are part of the CRF family, they are distinct from CRF and urocortin 1 (Rotzinger et al. 2010). The specific miRNAs of interest will be determined after the comprehensive study mentioned in section 3.1 of the project proposal is finished, as of now miR-34 and miR-127 seem promising targets. When the miRNAs of interest are determined they will be influenced either through lenti-/adeno-viral injections or through specific 'floxing' of animals and employing Cre-Lox recombinase to induce cell/tissue specific knockdowns of miRNAs. The actual choice will depend on the gene/microRNA in question, and whether there is a 'floxed' animal model available. To investigate the influence of decreased mitochondrial functioning on depression susceptibility a new mouse model with a gene trap insertion in the Ndufs4 allele will be used. This genetic alteration results in a premature termination of the coding sequence and deficiency of the NDUFS4 protein. This deficiency results in a decreased mitochondrial complex I and III activity of the electron transport chain, but the animals behavior remains normal under basal conditions.

The different animal models will be subjected to an acute stressor to investigate the immediate susceptibility to depression. Acute stress will be induced by using one of a variety of well-validated methods, all animals in the same experiment will receive one and the same stressor. We will assign both these genetically modified animals as well as WT mice randomly to the acute stress or control group. Control mice will be exposed to similar conditions compared to mice in the acute stress group, but without exposing them to the stressor. On these different animal models several behavioral tests will be executed after the stressor. After the last behavioral test, the animals are sacrificed and several biochemical and histological analyses will be performed. If robust changes are found, these studies are followed up by magnetic resonance imaging (MRI) to study brain structure and function, including DTI, rs-fMRI, and MRS. The neuroimaging will be executed on a different group of animals because it is known that isoflurane, that is used to anesthetize the animals, has an influence on mitochondrial function and on the stress response parameters. These animals will also undergo the different behavioral tests.

In order to determine the effect of the stressor on autonomic functions in the different animal models such as body temperature, as well as overall activity and sleep patterns, a telemetry sensor that can measure these will be implemented. This will both be WT as well as genetically modified animals. The sensor will be implemented in these animals minimal two weeks before the stress procedure in order for the animals to recover from surgery. In this time period it will also be possible to set a baseline for each animals before the stress experiment.

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or

Table 6. Short descriptions of acute stressors that can be used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Electrical foot shocks	The animals are placed in a skinner box where they will receive a series of inescapable unpredictable foot shocks with a duration of 20-30 min.	Severe
Physical restraint	The animals are placed in a plastic restrainer for 30 min to 1 h	Moderate
Tail suspension	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes.	Moderate
LPS injection	The animals will receive an intraperitoneal injection with lipopolysaccharide (for example E. Coli 055) to elicit an immune and stress reaction	Moderate

Table 7. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

Shortly after the last behavioral test the animals will be sacrificed. The sacrificing method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood, and several other organs will also be collected for additional analyses.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size was based and calculated using standard deviations from the test with the most variation from previous experiments. The effect size was $d=1.017$, giving a minimal number of animals of 11 per group. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 11 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly besides the stress paradigm. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the experimental- or control condition. This will be to strengthen the study design by limiting variation between groups due to experimenter bias and housing influences.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

Previous studies and experience in our lab using comparable acute stress-paradigms have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. This allows us to select the minimum of 10 animals per group however, we do not want to expose each animal to more than 3 behavioral tests post-stressor/non-stressor and we aim to do 4 to 6 behavioral tests. Therefore another group of animals has to be added for the additional behavioral tests, totaling at 20 animals per group (see table 8). The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of 10 animals per group, for details see table 8.

Table 8. Maximal number of animals necessary

Animal model	Number of animals per group				Total
	No-MRI		MRI		
	Control	Stress	Control	Stress	
Orexin KO / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
Ucn1 KO / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
Ndufs4def / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
MiRNA 1 / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
MiRNA 2 / WT	22 / 22	22 / 22	11 / 11	11 / 11	132
Total	110 / 110	110 / 110	55 / 55	55 / 55	660

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	660	Adult

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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The model we chose is one of the best validated animal models for stress-related psychopathology, and although it uses a acute stress exposure, it results in relative mild/moderate physical and psychological distress/harm compared some other stress-paradigms. The behavioral tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioral changes. Viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anesthesia (via 1.5% isoflurane inhalation) in order to minimize animal suffering. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that acute stress causes discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to

minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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.

Non applicable

Accommodation and care

F. Accommodation and care

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

[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?

G. Location where the animals procedures are performed

[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?

[] No > Continue with question I.

[X] Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

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

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

During viral injections, telemetry sensor implementation, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of 1.5% isoflurane inhalation. Otherwise no anesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

During the acute stress paradigm we will not preform anaesthesia or analgesia. When mitochondrial function will not be tested, and when perfusion will be used as a means of sacrifice, animals are first anesthetized by means of 1.5% isoflurane inhalation.

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

Stress will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, acute stress has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anaesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to acute stress, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful will be executed after the stressor to analyze differences in behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The stressors' nature is non-invasive, as well as relatively short (acute), in order to minimize physical- and mental distress, whilst still being able to induce differences between groups. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behaviour (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the acute stressors or have complications from surgery. Animals will constantly be monitored for signs of sickness, infection, excessive weight loss, or other signs of diminished wellbeing. If an animal during the experiment shows a body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or

veterinarian. Because of the non-invasive nature of the stressor, as well as the relatively short duration, and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The stressor is of a non-invasive nature, whereby the duration is not long enough that we expect severe stress to occur. The incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments. Based upon our previous experience with similar experiments with the orexin KO and Ucn1 KO mice, we expect to have less than 3% of the animals develop these criteria.

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 the animals experience from the acute stress paradigm is considered to be moderate or severe, depending on the particular stressor. The effect of the behavioral tests is expected to be mild to severe, depending on the behavioral test. The cumulative discomfort of the animals subjected to acute stress in combination with the behavioral tests is moderate or severe; for control mice this cumulative discomfort will be moderate or severe as well, as is classified is Annex VIII of the Directive 2010/63/EU.The severity of the discomfort depends on the stressor/behavioral test used, if the Porsolt swim test or electrical foot shocks are used it will be severe, if not and it the other stressor/behavioral tests the discomfort will be moderate. We expect that about 65% of the animals will experience severe discomfort and about 35% to have moderate discomfort.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without

these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number 4	Type of animal procedure Intervention experiment

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

The general design of this experiment is similar compared to the former three experiments. The same animal models will be used in order to study depression susceptibility with altered protein expression, either constitutively, via miRNA intervention or through specific floxing, and/or a decreased mitochondrial function. In this experiment the animals will be treated with different antidepressants. It has been known that different antidepressants have different side effects on for example mitochondrial functioning, some antidepressants can have a positive effect whilst others have a negative effect (Klinedinst and Regenold 2015).

The specific stress model that will be used in this intervention experiment depends on the effects observed from the former three experiments in this project. The stress model will be chosen after these experiments are finished. The stressor that yields the most robust effects on behavioral, biochemical, and immunohistochemical parameters will be used in this experiment. For the different animal models, different stress paradigms might be best suited to elicit depressive behavior or robust biochemical changes. For example, one animal model will give the best results with the chronic variable stress paradigm while another might give the best result to the social defeat stress. Animals will be exposed to the stress paradigm while being treated (with either treatment or vehicle).

The primary readout parameters will be the behavior of the animals in the different behavioral tests as well as the body weight, adrenal weight, and measurements of several biochemical parameters such as plasma corticosterone and adrenocorticotropic hormone levels, abundance of several proteins and/or mRNA in the brain, and mitochondrial function. These primary read out parameters are used because they are well-validated markers for depressive behavior, widely used, and easily measured. If these readout parameters establish a robust effect, neuroimaging will also be conducted. At the end of the experiments, behavioral data will be correlated to the biochemical data as well as neuroimaging data on brain structure and function (if neuroimaging is performed). In order to correlate biochemical, behavioral and neuroimaging data to each other the animals that undergo neuroimaging will also be behaviorally tested.

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

The aim of this experiment is to compare the genetically modified- or virally injected animals to WT littermates (non-genetically modified or control virally injected (injected with a virus containing a scrambled target sequence) target sequence respectively), by exposing them to a stressor followed by several standard behavioral tests devised to measure depression-related behavior. In addition to the stressor, in this experiment part of the animals will receive an antidepressant treatment. Two different antidepressants will be used depending on their effect on the mitochondria as

described above, as of now lithium chloride and fluoxetine would seem the bests to test at this moment. Animals will randomly be assigned to either the treatment or non-treatment group and all animals will be stressed.

Antidepressants will be given via a so called micro-osmotic pump. This is a small capsule that is inserted subcutaneous containing either an antidepressant or dissolvent. This method will be used because, although it includes surgery, it is less stressful for the animals compared to daily oral administration. At least a week before the stress paradigm, animals receive the micro-osmotic pump with either an antidepressant or dissolvent. This procedure will be done under general anesthesia (1.5 – 2% isoflurane inhalation) and the micro-osmotic pump will remain in the mouse for the duration of the experiment.

The animals that will receive a local viral injection will receive this in the same surgery as the micro-osmotic pump implantation. The viral injection is done while the animal is placed in a restraint / stereotactic apparatus, whilst the viral construct can be delivered through a thin syringe and needle.

After the surgery the animals will be subjected to either an acute stressor, a chronic stress paradigm, or social defeat stress, depending on the results from the representative experiments. The stress paradigm (and thus treatment period) that will be used is based upon the results of the previous experiments. This will also include several behavioral tests which measure behavior of the animals. For example the Porsolt swim test, tail suspension test, sucrose preference test, novelty suppressed feeing, open field test, Rotarod, splash test, and grip test. The behavioral tests that have a higher discomfort for the animals, Porsolt swim test and tail suspension test, will not be executed on the same animal. These two tests will be executed with two other milder behavioral tests. Short descriptions of these tests are included in table 9. Each behavioral test is executed once at the end of the stress paradigm.

Table 9. Short description of the different behavioral tests used.

Behavioral test	Short description	Discomfort level
Porsolt swim test	The animal is placed in a cylinder filled with water and is forced to swim for 6 minutes. The time the animals are inactive, i.e. not trying to escape is measured. This test can also be used as a stressor.	Severe
Sucrose preference test	In this test the animal will be faced with two bottles of water, one contains a sucrose solution whereas the other contains plain tap water. The ratio between drinking sucrose water and plain water is measured	Mild
Novelty suppressed feeding	The animal will be placed in a novel environment where a food pallet is placed in the middle. The time to approach and eat from the pallet is measured. This test can also be used as a stressor.	Mild
Open field test	The animal is placed in a novel environment where the general behavior is measured, e.g. locomotion speed, thigmotaxis, time spent in different quadrants, grooming behavior.	Mild
Rotarod	The animal is placed on a rotating drum in order to measure locomotor function. The time the animal spent on the drum before it falls off is measured.	Mild
Grip test	The animal is allowed to grab a metal grid or a triangular pull bar while being pulled backwards in the horizontal plane. The force applied to the grid or the bar just before the animal loses grip is recorded and measured.	Mild
Splash test	In this test the dorsal coat of a mouse is squirted with a 10% sucrose solution. The grooming behavior is recorded as an index of self-care and motivational behavior.	Mild
Tail suspension test	The animals are suspended by their tails with for example tape, in such a position that it cannot escape or hold on to nearby surfaces. Duration 10-15 minutes. The behavior of the animal trying to escape is measured.	Moderate

Shortly after the last behavioral test, the animals will be sacrificed. The sacrificing method is selected depending on post mortem experiments. If immunohistochemistry will be used the animals will be sacrificed by means of transcardial perfusion with phosphate buffered saline, if mitochondrial biochemical assays will be used the animals will be sacrificed by means of cervical dislocation. Mitochondrial function will be characterized post mortem in brain tissue by measuring functions such as mitochondrial respiration, complex I-IV activity, mitochondrial membrane potential, and ATP/ADP levels. The brain, blood, and several other organs will also be collected for additional analyses.

If clear and statistical significant behavioral and/or brain immunohistochemical or biochemical differences between groups emerge from these experiments, MRI experiments will be performed on a new group of animals to investigate brain structure and function. The MRI protocol includes DTI, rs-fMRI, and MRS and will be executed after the stress paradigm. Also one cluster of behavioral tests will be executed on these animals to correlate brain structure and function with behavior of the animals. These mice will be anesthetized using 1.5% isoflurane via inhalation. During the MRI experiments heart rate, breathing rate, and body temperature will be closely monitored and supported. Temperature is maintained at 37°C with warm airflow. Mice will be sacrificed directly after the MRI experiments by transcardial perfusion or through cervical dislocation while the mice are still under general anesthesia. The total MRI procedure is approximately two hours per animal.

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

To determine the minimal number of animals needed a G*Power calculation was employed with $P<0.05$ and a Power of 0.80. The effect size depends on the stressor that will be used during this experiment to be determined at a later time point. If the chronic variable stress paradigm will be used the effect size is $d=0.982$, giving a minimal number of animals of 14 per group. This example is chosen because this will then also be the maximal number of animals needed in this experiment, for the other stress paradigms fewer animals will be necessary. This calculation is applicable for all animal models in this project. Examination of previous work done with this specific paradigm, ruled out potential factors that would increase the population size, such as lethality and learning curve in animals.

The effect size for the MRI experiments is estimated to be $d=1.3$ from previous experiments requiring only 9 animals per group, however because we also will employ the same behavioral tests to these animals the minimum number of animals needed for the MRI experiment would also be 14 animals per group.

Furthermore, all groups will be housed at comparable conditions and treated similarly. Also, both genetically modified animals as well as WT littermates will randomly be assigned to either the one of the treatment or control conditions. This will be to strengthen the study design by limiting variation between groups due to experimenter bias and housing influences. In previous studies in our lab we observed that the subcutaneous implantation of the micro-osmotic pumps per se did not cause any mortality or extreme discomfort for the animals.

B. The animals

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

We will use mice (Mus musculus) in our experiments. We need animals with a complex enough physiology, including a developed nervous system, in order to test our hypotheses. A human test population would prove too heterogeneous in their genetic background, environmental experiences, and for instance the possible prevalence of (undiagnosed) psychiatric disorders. Rodents are the lowest species available that are suitable for our experiments, and also allow some comparison to the human physiology.

Standard C57BL/6J or FVB/NJ as WT control mice will be used (depending on the background of the genetically modified animal tested); they are well studied, documented, and tested in the past, and have been genetically modified with success. Another advantage is the high availability of genetically modified variants of the C57BL/6J and the FVB/NJ mouse strains. We will test three genetically modified animal models; the orexin null mice (orexin KO), urocortin 1 null mice (Ucn1 KO), and Ndufs4 deficient (Ndufs4def) mice. The orexin KO (Mochizuki et al. 2004) and Ucn1 KO (Vetter et al. 2002) mice are both from a C57BL/6J background, whereas the Ndufs4def mice are from a FVB/NJ background. The three genetically modified mouse lines will further deepen our insight into the significance of altered peptide homeostasis as well as decreased mitochondrial function in controlling the adaptive stress response.

In addition to the proposed animals described above, we are also planning to test the effect 2 specific microRNAs. The specific miRNAs will be based on the results of the comprehensive study mentioned earlier in the project proposal. The number of animals needed for each experiment is set to a minimum but still high enough to determine statistical differences.

We will use only male animals because it has been shown that ovarian hormones have a powerful effect on the stress response (Viau and Meaney 1991, Wood and Shors 1998, Ter Horst et al. 2009, Goldstein et al. 2010). Because of this, males with a stable hormonal cycle show less variation compared to females with the fluctuating hormonal cycle. Once we establish our project aims, we will consider to perform similar studies in female animals in a follow up study (for this purpose a new application will be prepared). Animals will be tested in the young adult life phase for we aim to investigate the effects in the adult organism, not during development. Also after puberty the hormonal cycle of the males is stable reducing variation keeping the number of animals to a minimum.

At this point in time it is still unsure what specific antidepressants are the best option for this experiment. This is because first all three experiments must be executed and analyzed to determine the best suitable stressor per animal model. The influence of different antidepressants on mitochondrial function is a new and rapidly changing field of research, some antidepressants have a positive effect on mitochondrial function whilst others have a negative effect. As of now the best options would be to use lithium chloride which have a positive effect on the mitochondria and fluoxetine which have a negative effect on the mitochondria. A third treatment group is the control group where the micro-osmotic pumps will be loaded only with vehicle as a control. Depending on the stressor that will be chosen for this experiment, the maximum number of animals that is necessary is 14 animals per group (if chronic variable stress is used).

Previous studies and experience in our lab using comparable stress-paradigms as well as micro pump injections have shown that lethality can be kept to a bare minimum. Therefore, there will be no need to compensate for any potential deaths during testing. Depending on the stressor that will be used the minimum number of animals per subgroup will be 14. **Depending on the number of behavioral tests that yielded interesting results from the experiment that this intervention experiment is based upon, we might need only one cluster of animals. If 3 or fewer**

behavioral tests are interesting to investigate only one cluster of animals will be used, if 4 or more behavioral tests will be executed we will have to multiply the number of animals by two as described in the previous animal procedures, totaling 28 animals per group (see table 10). This is because we do not want to expose each animal to more than 3 behavioral tests post-stressor. The reason for this is that the behavioral tests themselves are stressful and are serious confounds in all stress experiments. The compromise to have three tests per animal is well-accepted to minimize the number of animals on the one hand while on the other hand minimizing the confounding the effect of the behavioral test on stress measures.

If robust changes are found in behavior or biochemical analysis neuroimaging will be used on a separate group of stressed/non-stressed mice. These animals will also be behaviorally tested, however not for all tests previously described. The three behavioral tests that yielded the most robust effects will be used. This necessitates the use of maximal 14 animals per group, for details see table 10.

Table 10. Maximal number of animals necessary

Animal model	Number of animals per group						Total
	No-MRI Stress			MRI Stress			
	Anti-1	Anti-2	Veh.	Anti-1	Anti-2	Veh.	
Orexin KO / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
Ucn1 KO / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
Ndufs4def / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
MiRNA 1 / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
MiRNA 2 / WT	28 / 28	28 / 28	28 / 28	14 / 14	14 / 14	14 / 14	252
Total	140 / 140	140 / 140	140 / 140	70 / 70	70 / 70	70 / 70	1260

Species	Origin	Maximum number of animals	Life stage
Mouse	B2. (In een geregistreerd fok- of afleveringsbedrijf in de EU, inclusief Nederland, geen apen)	1260	Adult

C. Re-use

Will the animals be re-used?

C. Re-use

[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.
Research strongly suggests that the human psychopathology cannot be studied in lower vertebrates species, thus the use of rodents and primates has been widely accepted within the scientific community. In vitro studies will provide us with viable targets to test, however in order to accurately predict the mechanics by which these genes function in humans, we will need to test them on other vertebrates first. Rodents represent a useful in vivo model with an intact, functioning neuronal network (neurons, glial cells, vascular system). Rodents are often used to model human psychopathology, there is at the moment and to the best of our knowledge no alternative to the use of rodent animal models to study the stress-adaptation response that is comparable to the human situation.

Reduction.
Upon planning the experiments all efforts have been made to minimize the number of animals used in this study. Experience from previous similar experiments and G*power analysis show that the requested number of mice is the very lowest number of mice that would be needed to test our hypothesis. The limited use of invasive procedures and experience in our lab should minimize lives lost due to procedures, infections, etc. Also, all animals are randomly appointed to the different groups.

Refinement.
Animals will be housed together with littermates in suitable cages with ad libitum access to food and water. All experiments were designed and will be carried out to minimize the suffering of the animals. The behavioural tests are selected to induce as little stress and suffering as possible, whilst still resulting in reliable data on depression-like behavioural changes. Viral injections, micro-osmotic pump implementation, telemetry sensor implementation, perfusion as a method of sacrifice, and MRI will all be performed under general anaesthesia (via 1.5% isoflurane inhalation) in order

to minimize animal suffering. Also, 3 days post-surgery the animals will receive analgesia. Humane endpoints will be selected, to prevent unnecessary or excessive suffering.

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

We are aware that stress and the surgery cause discomfort to animals. However, stress induction is inevitable to investigate the neuronal functions and circuitries, as well as other biological processes involved in the stress adaptation and investigate susceptibility to stress related disorders. In order to minimize suffering and number of mice, animals will be housed together with littermates in suitable cages with ad libitum access to food and water. To minimize pain and distress of the animals during the MRI experiments, as well as during the viral injections, micro/osmotic pump placement, and telemetry sensor implementation, mice will be anesthetized by means of inhalation of 1.5% isoflurane. After surgery the animals will be treated with analgesics to alleviate the initial pain from the wound.

Expertise on the proposed experiments is available as well as required equipment and accommodation for the animals. This allows the procedures to be performed efficiently and with the least amount of distress for the animals. However, should any animal during the experiment show any sign of an unnecessary large amount of distress, humane endpoints will be implemented. After consultation with a veterinarian or an animal welfare expert the animal will be removed from the experiment and sacrificed to end suffering.

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?

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

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

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

During viral injections, telemetry sensor implementation, micro-osmotic pump insertion, perfusion as a method of sacrifice, or MRI experiments animals will be anesthetized by means of isoflurane inhalation of 1.5%. Otherwise no anaesthesia will be used, as this will influence any stress response parameters as well as the mitochondrial function. Also, up to three days post-surgery lidocaine will be applied cutaneous as an analgesic.

I. Other aspects compromising the welfare of the animals

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

Stress will cause discomfort for the animals. To investigate neuronal circuitries/functions involved in stress adaptation, acute stress has to be applied on a part of the mice. Furthermore, animals will also be subjected to several behavioral tests that leads to distress for the animals. During the MRI procedure and surgery, animals will be anesthetized using 1.5% isoflurane. Side effects of isoflurane include respiratory depression and a decrease in heart rate and blood pressure. When necessary, the level of anaesthesia can be adjusted very rapidly. No other adverse effects are expected.

Explain why these effects may emerge.

A group of animals will be exposed to a stressor, which is known to have effects on the welfare of the animals as seen by behavioral changes. The animals will develop distress, which will be visible physically or behaviorally, as has been shown in past studies that used a similar design. Also, behavioral tests that can be stressful are executed after the stress paradigm to determine their behavior. During the MRI protocol as well as surgery, mice are anesthetized with isoflurane, where respiratory depression is not an uncommon side effect.

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

The stress paradigms planned in this experiment are widely used in the research towards the stress response and stress related behaviors such as depressive behavior. Besides the stressors inflicted for the experiment, all efforts have been made in order to minimize the suffering of the animals in this experiment. We will house animals together, and provide food and water ad libitum, and keep track of their weight, body temperature, respiration frequency, general health, and behaviour (such as grooming), in order to determine whether the animals experience inappropriate levels of distress. If animals are suffering unnecessarily, we will confer with animal caretakers, the animal welfare expert, and a veterinarian, and apply humane endpoints if necessary.

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.

Some animals may experience more stress than anticipated for the stressors or have complications from surgery. Animals will constantly be monitered for signs of sickness, infection, excessive weight loss, or other signs of diminished wellbeing. If an animal during the experiment shows a

body weight loss of more than 15%, piloerection, or a significant decrease of grooming, this will be considered as a sign of unnecessary distress. When this is observed, the animal will be removed from the experiment and immediately terminated upon consultation of an animal caretaker and/or veternarian. Because of the non-invasive nature of the stressor, as well as the relatively short duration, and also based on previous experiments it is unlikely that the humane endpoints will need to be applied.

Indicate the likely incidence.

The incidence of extreme discomfort in the animals is minimal and unlikely, as known from previous similar experiments and experience in our lab with micro-osmotic pump surgery as well as dietary interventions. We expect to have less than 3% of the animals develop these criteria.

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 the animals experience from the stress paradigm is considered severe. The effect of the behavioral tests is expected to be mild to severe, depending on the behavioral test. The cumulative discomfort of the animals in this experiment is severe (100%) as is classified is Annex VIII of the Directive 2010/63/EU.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

After the experiment animals are sacrificed in order to study several physiological processes involved in the stress response. For most of these measurements, post mortem biological material is needed. This will be investigated with several biochemical and histological techniques. Without these techniques, we would not be able to determine if the stress and/or genetic modifications have any effects on a cellular and molecular level. This would prevent us from coupling the potential changes in behavior to actual changes in physiology.

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

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

[X] Yes

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD103002016481
Bijlagen
1

Datum 18 mei 2016
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 18 maart 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Unraveling the underlying mechanisms of depression" met aanvraagnummer AVD103002016481. Wij hebben uw aanvraag beoordeeld.

Op 25 april 2016 heeft u uw aanvraag aangevuld. U heeft op de vragen van de CCD gereageerd. In uw antwoord heeft u uitgelegd waarom het wenselijk is om alle gedragstesten te gebruiken in de proeven 3.4.4.1-3.4.4.3 en heeft u aangegeven in dierproef 3.4.4.4 rekening te gaan houden met de uitkomsten uit de vorige proeven. U heeft ook het aantal muizen te gebruiken als resident in dierproef 3.4.4.2 gehalveerd. U heeft uw aanvraag aangepast en een nieuwe versie van de formulieren naar ons toe gestuurd.

Bij de behandeling van uw aanvraag heeft de CCD opgemerkt dat enige voorzichtigheid is geboden bij directe vertaling van gedragsstudies bij dieren naar effecten, zoals depressie, bij de mens.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. De voorwaarde betreffende het afstemmen van de go/no-go momenten met de IvD is gesteld in het kader van de 3V's, om te voorkomen dat dat dieren onnodig worden gebruikt in het geval dat de experimenten niet de verwachte resultaten opleveren. De algemene voorwaarde betreffende artikel 10, lid 1 sub a van de wet wordt gesteld bij vergunningen met een langere looptijd. Dit om te voldoen aan datgene wat volgt uit dit artikel. U kunt met uw project "Unraveling the underlying mechanisms of depression" starten. De vergunning wordt afgegeven van 1 juni 2016 tot en met 31 mei 2021. Deze termijn is anders dan in uw aanvraag, omdat de looptijd van de vergunning maximaal 5 jaar is.

Overige wettelijke bepalingen blijven van kracht.

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

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie RU Dec gevoegd. Dit advies is opgesteld op 17 maart 2016. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie, nemen wij over, inclusief de daaraan ten grondslag liggende motivering. In aanvulling op dit advies stelt de CCD twee algemene voorwaarden zoals in de vergunning genoemd.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

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

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

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

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

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

Centrale Commissie Dierproeven
namens deze:

[redacted]

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

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

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

deze projectvergunning voor het tijdvak 1 juni 2016 tot en met 31 mei 2021, voor het project "Unraveling the underlying mechanisms of depression" met aanvraagnummer AVD103002016481, volgens advies van Dierexperimentencommissie RU Dec. De functie van de verantwoordelijk onderzoeker is Onderzoeker in opleiding. Voor de uitvoering van het project is Instantie voor Dierenwelzijn verantwoordelijk.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 18 maart 2016
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 25 april 2016;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 25 april 2016;
c Advies van dierexperimentencommissie d.d. 17 maart 2016, ontvangen op 18 maart 2016.
d De aanvullingen op uw aanvraag, ontvangen op 25 april 2016

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Chronic variable stress paradigm	Muizen (Mus musculus) / Orexin KO/WT; Ucn1 KO/WT; Ndufs4def/WT; MiRNA 1/WT; MiRna 2/WT	900	75,00% Ernstig 25,00% Matig	
3.4.4.2 Chronic Social defeat stress	Muizen (Mus musculus) / Gelijk aan dierproef 3.4.4.1 en BALB/cJ	1080	37,50% Ernstig 62,50% Matig	
3.4.4.3 Acute stress paradigm	Muizen (Mus musculus) / Gelijk aan dierproef 3.4.4.1.	660	65,00% Ernstig 35,00% Matig	
3.4.4.4 Intervention experiment	Muizen (Mus musculus) / Gelijk aan dierproef 3.4.4.1.	1260	100,00 % Ernstig	

Voorwaarden

Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen

In dit project worden dierproeven toegepast waarbij die vallen in de categorie ernstig volgens artikel 10b van de wet en wordt daarom voorzien van beoordeling achteraf.
Deze beoordeling zal uiterlijk mei 2022 plaatsvinden. Er zal dan beoordeeld worden of de doelstellingen van het project werden bereikt. Daarnaast wordt bekeken of de schade die de dieren hebben ondervonden, het aantal en soorten proefdieren en de ernst de dierproeven conform de vergunning waren.

Algemene voorwaarden:
De vergunning wordt afgegeven onder de voorwaarde dat de go/no-go momenten worden afgestemd met de IvD.

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

Weergave wet- en regelgeving

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

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

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

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

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

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