	Inventaris Wob-verzoek W16-11S								
		wordt verstrekt				**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS2016405								
1	Aanvraagformulier				x		x	x	
2	Projectvoorstel				x		x	x	
3	Niet-technische samenvatting	x							
4	Bijlage beschrijving dierproeven 1				x		x	x	
5	Bijlage beschrijving dierproeven 2				x		x	x	
6	Bijlage beschrijving dierproeven 3				x		x	x	
7	Bijlage beschrijving dierproeven 4				x		x	x	
8	Bijlage beschrijving dierproeven 5				x		x	x	
9	DEC-advies				x		x	x	
10	Ontvangstbevestiging				x		x	x	
11	Advies CCD		x						x
12	Beschikking en vergunning				x		x	x	
13	Mail terugkoppeling DEC 25-2-2016				x		x	x	

28 JAN. 2016

Centrale Commissie Dierproeven

AVD 1050020164 05

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

1 Gegevens aanvrager

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

☒ Ja > *Vul uw deelnemernummer in* 10500
☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	Rijksuniversitiet Groningen
Naam van de portefeuillehouder of diens gemachtigde	[redacted]
KvK-nummer	1179037

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	A. Deusinglaan 1 [redacted]
Postbus	
Postcode en plaats	9713 AV GRONINGEN
IBAN	
Tenaamstelling van het rekeningnummer	

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	PhD student
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

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

2 Over uw aanvraag

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

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1 - 12 - 2015
		Einddatum	1 - 12 - 2020
3.2	Wat is de titel van het project?	Train the sedentary brain: Move smart to reduce the risk of dementia	
3.3	Wat is de titel van de niet-technische samenvatting?	Train het inactieve brein: Slim bewegen om de kans op dementie te verminderen	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC-RUG
		Postadres	A. Deusinglaan 1, [redacted]
		E-mailadres	secrdec.umcg@umcg.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1727 Lege

☐ Wijziging € Lege

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

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☒

6 Ondertekening

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

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	[redacted]
Functie	[redacted]
Plaats	GRONINGEN
Datum	27-01-2016
Handtekening	[redacted]

Format
Projectvoorstel dierproeven

- Dit format gebruikt u om uw projectvoorstel van de dierproeven te schrijven
- Bij dit format hoort de bijlage Beschrijving dierproeven. Per type dierproef moet u deze bijlage toevoegen.
- Meer informatie over het projectvoorstel vindt u op de website www.zbo-ccd.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Vul uw deelnemernummer van de NVWA in.	10500
1.2	Vul de naam van de instelling of organisatie in.	Rijksuniversiteit Groningen
1.3	Vul de titel van het project in.	Train the sedentary brain: Move smart to reduce the risk of dementia

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

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

3 Algemene projectbeschrijving

3.1 Achtergrond

Licht het project toe. Beschrijf de aanleiding, de achtergrond en de context. Besteed aandacht aan de bij vraag 2 aangekruiste categorieën.

- Geef in geval van 'wettelijk vereiste dierproeven' aan welke wettelijke eisen (in relatie tot beoogd gebruik en markttoelating) van toepassing zijn.
- Geef in geval van 'routinematige productie' aan welk(e) product(en) het betreft en voor welke toepassing(en).
- Geef in geval van 'hoger onderwijs of opleiding' aan waarom in dit project, in relatie tot het opleidingsprogramma en eindtermen, is gekozen voor dierproeven.

Background

Alzheimer's disease (AD) is the main cause of dementia.[1] AD is characterized by a progressive decline in several cognitive domains including learning, memory and language. The cause of AD is still largely unknown, and AD is viewed as a multifactorial and complex disease with aging as the main risk factor.[2,3,4] The only universal genetic risk factor for AD is the Apo-lipoprotein e4 (APOEe4) allele. Carriers of this allele have a significantly higher risk of developing AD. In contrast, carriers of the APOEe3 allele do not have an increased risk of developing AD. As compared to the homozygote APOEe3 genotype, heterozygous and homozygous APOEe4 carriers have a three to thirteen fold increased risk of developing AD respectively.[5]

Next to this genetic risk factor, physical inactivity has been recognized as an environmental (life style) risk factor for AD.[6-8] Of note, APOEe4 carriers are found to be particularly at risk with regard to the consequences of inactivity: Sedentary behavior moderates Amyloid plaque formation, a pathological hallmark of AD, in APOEe4 carriers.[9]

Optimally stimulating physical activity in humans to battle the development of AD is being researched intensively today in a national consortium funded by ZonMW Deltaplan Dementie, of which this project is the preclinical part. Clinical research by our partners takes place in individuals prone to develop AD or diagnosed with early signs of AD. A recent study performed in Groningen suggests that strength exercise, in combination with endurance exercise, is more successful in slowing down cognitive decline in AD patients than endurance exercise alone.[10] Use of endurance exercise interventions is currently common practice. Therefore, additional research is warranted to optimize the exercise protocols. However, the development of optimal exercise interventions is time consuming and in need of being directed by underlying neurobiological and neurobehavioral processes. The relation between specific types of exercise or physical inactivity and brain processes can best be studied in mice, as proposed in this project.

In addition to physical activity, cognitive stimulation may also reduce the progression of AD.[6] In mice, cognitive stimulation can be mimicked by providing a challenging environment, called "enriched environment". [11-13] Therefore, next to physical activity, cognitive stimulation by way of an enriched environment will be used as an intervention therapy to battle the development of AD. As episodic memory is one of the first types of memory negatively affected by AD, we specifically focus on the use of episodic memory tasks next to gold standard tasks in mouse studies on cognition.[14]

Motivation

A clear view of which type of physical activity (type of exercise) can be beneficial to counter AD will help to develop intervention therapies that prevent or slow down the progression of AD. This is of particular importance for the group of people most likely to develop AD, the APOEe4 carriers. Similarly, it is important to know in more detail what the effects of inactivity are on brain health and functioning, knowing that inactivity is a crucial risk factor for developing AD. We have seen that next to physical activity and inactivity, cognitive stimulation is beneficial for proper brain functioning. Therefore, combining physical and cognitive stimulation could be most beneficial in slowing down the progression of AD. Our mouse studies will help to direct the ongoing and planned exercise and cognitive stimulation projects with (early) AD patients in our national consortium, and will gain insight in the underlying brain mechanisms related to the selected intervention therapies.

Translational value

To examine the relationship between physical activity (exercise), physical inactivity and cognitive stimulation on brain functioning in slowing down AD pathology, we will use aging mice as well as APOE and AD mouse models. With this approach, we have the three major risk factors of developing AD under study: age, physical/mental inactivity, and the APOEe4 allele. Mouse studies provide an opportunity to disentangle the different risk factors and their effect on cognition, AD pathogenesis and brain health in general. These studies should provide a clearer view on the impact of the various interventions at the level of the brain and cognitive performance. The results will be translated into suggestions for more optimal physical/mental intervention therapies for humans in risk of developing AD.

The proposed experiments are part of a national consortium consisting of four research groups from the University of Groningen, the University Medical Center Groningen, the VU University Amsterdam and the Radboud University Medical Center. This consortium is funded by the ZonMW in the Memorabel program, which is part of the Deltaplan Dementie. We focus on pre-clinical research, whereas the other groups try to answer similar research questions by performing exercise intervention studies in elderly, APOEe4 carriers and AD-patients. Our animal studies have been designed so that they can be compared to these human studies in terms of exercise interventions and outcome measures. Cognitive functions that are tested in our animals are matched to those tested in humans. Moreover, markers for which the other groups will test in blood of elderly and patients, will also be tested for in our animal studies. In this way, we can translate our results to the human situation in a rather direct manner and link mechanisms uncovered in our studies to the outcomes of the human studies.

References:

1. Jellinger, K., Danielczyk, W., Fischer, P. & Gabriel, E. Clinicopathological analysis of dementia disorders in the elderly. *J. Neurol. Sci.* **95,** 239–258 (1990).
2. Association, A. Alzheimer ' s disease facts and figures. *Alzheimer's Dement.* **11,** 332–384 (2015).
3. Bertram, L. & Tanzi, R. E. Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses. *Nat. Rev. Neurosci.* **9,** 768–778 (2008).
4. Corder, E. H. *et al.* Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science* **261,** 921–923 (1993).
5. Raber, J., Huang, Y. & Ashford, J. W. ApoE genotype accounts for the vast majority of AD risk and AD pathology. *Neurobiol. Aging* **25,** 641–650 (2004).
6. Barnes, D. E. & Yaffe, K. The projected effect of risk factor reduction on Alzheimer's disease prevalence. *Lancet Neurol.* **10,** 819–828 (2011).
7. Biswas, A. *et al.* Sedentary Time and Its Association With Risk for Disease Incidence, Mortality, and Hospitalization in Adults. *Ann. Intern. Med.* **162,** 123 (2015).
8. Dogra, S. & Stathokostas, L. Sedentary Behavior and Physical Activity Are Independent Predictors of Successful Aging in Middle-Aged and Older Adults. *J. Aging Res.* **2012,** 1–8 (2012).
9. Bugg, J. M. Exercise Engagement as a Moderator of the Effects of Genotype on Amyloid Deposition. *Arch. Neurol.* **69,** 636 (2012).
10. Bossers, W. J. R. *et al.* A 9-Week Aerobic and Strength Training Program Improves Cognitive and Motor Function in Patients with Dementia: A Randomized, Controlled Trial. *Am. J. Geriatr. Psychiatry* (2015). doi:10.1016/j.jagp.2014.12.191
11. Fabel, K. *et al.* Additive effects of physical exercise and environmental enrichment on adult hippocampal neurogenesis in mice. *Front. Neurosci.* **3,** 50 (2009).
12. Bonenfant, D., Nguyen, A. Le & Aumont, A. Untangling the Influences of Voluntary Running , Environmental Complexity , Social Housing and Stress on Adult Hippocampal Neurogenesis. **9,** (2014).
13. Lambert, T. J., Fernandez, S. M. & Frick, K. M. Different types of environmental enrichment have discrepant effects on spatial memory and synaptophysin levels in female mice. *Neurobiol. Learn. Mem.* **83,** 206–216 (2005).
14. Dubois, B. *et al.* Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol.* **9,** 1118–1127 (2010).

3.2 Doel

Beschrijf de algemene doelstelling en haalbaarheid van het project.

- In het geval het project gericht is op één of meer onderzoeksdoelen: op welke vra(a)g(en) dient dit project antwoord(en) te verschaffen?
- In geval het een ander dan een onderzoeksdoel betreft: in welke concrete behoefte voorziet dit project?

*Objective*s

- The first objective is to examine the relationship between physical (in)activity and brain health and cognition in the context of aging and AD.
- The second objective is to identify which type of exercise intervention, including combined exercise and cognitive stimulation, is best suited to counteract age-related and AD-related cognitive decline.

Six research questions will be answered:

1. How do physical inactivity, strength exercise and endurance exercise affect brain health and cognitive functioning in aging mice?
2. How do physical activity and cognitive stimulation interact to affect brain health and cognitive functioning in aging mice?

3. How do physical inactivity, strength exercise and endurance exercise interact with the genetic risk factor APOEe4 to affect brain health and cognitive functioning in human APOEe4 mice?
4. How do physical activity and cognitive stimulation interact with the genetic risk factor APOEe4 to affect brain health and cognitive functioning in human APOEe4 mice?

5. How do physical inactivity, strength exercise and endurance exercise affect brain health and cognitive functioning in the context of AD pathology in (amyloid) AD mice?
6. How do physical activity and cognitive stimulation affect brain health and cognitive functioning in the context of AD pathology in (amyloid) AD mice?

Achievability

To reach these objectives we will investigate the effects of strength exercise, endurance exercise, inactivity, cognitive stimulation, and combinations of these interventions on brain health and cognition in healthy (aged) mice, mouse models of AD and the APOEe4 genotype. Moreover, we will investigate cellular and molecular mechanisms that can explain the link between the primary functional output parameters (between cognitive functioning and cardiovascular fitness or strength).

Local facilities enable us to breed the APOEe4 and AD-mouse models, so that we can time the arrival of cohorts of mice as needed. Furthermore, our research group is experienced in performing both (episodic) learning and memory studies and exercise interventions in rodents.[1-3] Therefore we do not expect any major technical difficulties. Moreover, we have recently developed novel strength exercise methods and a novel inactivity method in mice.[4] Further studies are needed to ensure our strength exercise methods and inactivity methods are suitable for aged and AD-model mice. (Appendix 1) Similarly, tests for episodic memory need to be optimized too. (Appendix 2)

References:

1. [redacted]
2. [redacted]
3. [redacted]

4. [redacted]

3.3 Belang

Beschrijf het wetenschappelijk en/of maatschappelijk belang van de hierboven beschreven doelstelling(en).

General scientific and clinically relevance
Voluntary running in rodents, a form of endurance exercise, has been investigated quite extensively in pre-clinical studies and with respect to its effects on the brain.[1] Strength exercise has received far less attention.[2-4] It is of scientific relevance to reveal the mechanisms that underlie the effects of strength exercise on the brain: This is needed in order to fully understand the relationship between physical activity, which is more than just endurance exercise, and brain health. Clinical intervention studies employ exercise interventions which contain strength exercise components in both elderly an AD patients.[5] Animal studies can be used to optimize these interventions (for specific patient groups) and to elucidate the mechanisms via which they affect the brain.

There is a lack of knowledge on the mechanisms that underlie detrimental effects of inactivity on cognition and the brain in elderly and especially in AD patients.[6] Again, to elucidate the mechanisms that underlie the effects of inactivity on the brain is of scientific relevance as we do not know how inactivity affects the brain. One open question here is whether inactivity affects the brain by inhibiting effects on the same mechanisms and pathways that (endurance) exercise stimulates. Increasing our understanding on the effects of inactivity can help to design interventions that counter or prevent detrimental effects of inactivity in a clinical setting such as elderly homes or the hospital.

As is mentioned in part 3.1, studies performed in healthy mice show that cognitive stimulation and physical activity induce differential (neurogenic) effects that are thought to be complementary. This hypothesis has not been tested directly, nor in the context of APOE4 and AD. To understand how these environmental factors interact is of importance because interactions are likely to occur in any environment which is more complex than a standard laboratory setting, such as the home environment or a nursery home.

Genotype x Environment interactions
A deeper understanding of how environmental factors influence cognition and brain health can help to improve construct validity of transgenic animal models of complex diseases like AD. Since AD arises through interactions of environmental and genetic risk factors, animal models of AD should include both genetic (APOEe4) and environmental risk factors (e.g. inactivity) too. Novel methods we develop to induce physical inactivity can help improve the validity of any transgenic rodent model of a complex disease for which physical inactivity is an environmental risk factor. [7] Therefore an inactivity method is of scientific relevance for more than only AD mouse models.

The effects of inactivity and strength exercise have not been investigated in transgenic human APOEe4 mice and AD mouse models. The effect of endurance exercise has been investigated in rodent models, but very scarcely in APOEe4 mice.[1,8] It is of scientific relevance to investigate which mechanisms underlie the interaction between the APOEe4 genotype and exercise and inactivity that have been observed in humans, this increases our understanding of the pathogenesis of sporadic AD.[9] Clinically, this knowledge can be used to pinpoint which patients are likely to benefit from exercise interventions (e.g. all elderly or only APOEe4 carriers).

Episodic memory

We will investigate the effects of exercise or inactivity on a specific form of memory: Episodic memory. In AD patients episodic memory is affected in early stages of the disease.[10] How this form of memory is specifically affected by (in)activity and the APOE4 genotype in sporadic AD patients is not known. Research shows that the APOE genotype and neurotrophic factors that are affected by endurance exercise interact to influence episodic memory.[11] Knowledge of how episodic memory is affected by physical (in)activity could prove valuable in devising early intervention strategies for AD patients and people at risk of developing AD (marked by a decline in episodic learning and memory). Testing of this specific form of memory is not often performed in AD mouse models and has not been performed in APOE4 mice, testing episodic memory in APOEe4 and AD mouse models could help to elucidate possible causes of the early decline in episodic learning and memory in sporadic AD.

References:

[1] Intlekofer, K. A. & Cotman, C. W. Exercise counteracts declining hippocampal function in aging and Alzheimer ' s disease. *Neurobiol. Dis.* **57,** 47–55 (2013).

[2] Seo, D., Lee, S., Kim, N. & Ko, K. Humanized animal exercise model for clinical implication. *Pflügers Arch. …* **466,** 1673–1687 (2014).

[3] Konhilas, J. P. *et al.* Loaded wheel running and muscle adaptation in the mouse. *Am. J. Physiol. Heart Circ. Physiol.* **289,** H455–65 (2005).

[4] Suijo, K. *et al.* Resistance exercise enhances cognitive function in mouse. *Int. J. Sports Med.* **34,** 368–75 (2013).

[5] Bossers, W. J. R. *et al.* A 9-Week Aerobic and Strength Training Program Improves Cognitive and Motor Function in Patients with Dementia: A Randomized, Controlled Trial. *Am. J. Geriatr. Psychiatry* (2015). doi:10.1016/j.jagp.2014.12.191

[6] Brown, B. M., Peiffer, J. J. & Martins, R. N. Multiple effects of physical activity on molecular and cognitive signs of brain aging: can exercise slow neurodegeneration and delay Alzheimer's disease? *Mol. Psychiatry* **18,** 864–874 (2013).

[7] Hunter, D. J. Gene-environment interactions in human diseases. *Nat. Rev. Genet.* **6,** 287–298 (2005).

[8] Nichol, K., Deeny, S. P., Seif, J., Camaclang, K. & Cotman, C. W. Exercise improves cognition and hippocampal plasticity in APOE 3 4 mice. *Alzheimer's Dement.* **5,** 287–294 (2009).

[9] Head, D. *et al.* Exercise Engagement as a Moderator of the Effects of APOE Genotype on Amyloid Deposition. *Arch. Neurol.* **69,** 636–43 (2012).

[10] Dubois, B. *et al.* Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol.* **9,** 1118–1127 (2010).

[11] Ward, D. D. *et al.* APOE and BDNF Val66Met polymorphisms combine to influence episodic memory function in older adults. *Behav. Brain Res.* **271,** 309–315 (2014).

3.4 Onderzoeksstrategie

3.4.1 Geef een overzicht van de algemene opzet van het project (strategie).

In order to perform the experiments designed to meet the objectives specified in part 3.2 we first need to meet the following prerequisites:
1. A working method to induce strength exercise in (aged) mice and AD model mice.
2. A working method to induce inactivity in (aged) mice and AD model mice.
3. A working cognitive test battery including episodic memory tests, which can be performed successfully by (aged) mice and AD mice.

Previous work

We are currently comparing a number of strength exercise and inactivity methods in order to select the most effective methods. These studies are performed in healthy adult mice. Two of the models used, burrowing and resistance running, have been shown to improve grip strength. Muscle tissue from these mice is being analysed to determine which of these models would be the best strength exercise method.[1] We were able to induce inactivity by using a lid in which the mice could not climb in and by using different cage sizes.[1] This inactivity method is being characterized further at the moment by determining its effects on grip strength, stamina and muscle tissue. A custom made episodic memory test, the automated Time-Place learning test, was

tested in adult mice in a single technical pilot study.[2]

Project strategy
We will start by further testing strength exercise and inactivity methods in healthy adult mice, aged mice and AD mice. This is necessary to ensure they can be used in aged and AD mice. (Appendix 1) We need to test and optimize our (custom made) automated Time-Place learning set-up and the What-Where-When-Which test (WWWW-test) for episodic memory in adult mice, aged mice and AD mice too (Appendix 2). Once these methods have been optimized, we can commence with the main experiments.

Main experiments. (Appendix 3, 4 and 5, can be performed in parallel)
Adult and aged mice, human-APOEe4 mice and human-APOEe3 control mice and AD mice will be submitted to intervention methods that induce inactivity, strength exercise, endurance exercise, cognitive stimulation (Enriched environment) and combinations of exercise and cognitive stimulation. The interventions will last for 10-12 weeks. After this period, the mice will be submitted to a set of cognitive tests (including episodic memory tests). Physical fitness tests will be used to measure physical fitness and motor functioning of the same animals to confirm that the (in)activity methods had the desired physical fitness effects.

Similar groups of mice will be undergoing the same interventions, but will not undergo cognitive testing. These mice will be sacrificed and their brain tissue, blood, cerebrospinal fluid and muscles will be analysed in the lab to determine how (in)activity interventions affect a number of neuro-molecular output parameters. Mice that did undergo cognitive testing cannot be analysed this way because of the confounding effects of the cognitive testing on a number of outcome parameters in the brain: Both exercise and learning and memory tests influence the same neuro-molecular mechanisms (i.e. neurogenesis and Brain-Derived Neurotrophic Factor signalling in the hippocampus), making it impossible to separate the effects of exercise and the memory tests.

References:
[1] [redacted]
[2] [redacted]
[3] Bour, A. *et al.* Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks. *Behav. Brain Res.* **193,** 174–82 (2008).

3.4.2 Geef een overzicht op hoofdlijnen van de verschillende onderdelen van het project en de daarbij gebruikte type(n) dierproef of dierproeven.

The project is divided in two smaller studies (Appendix 1 and 2) and three main studies (Appendix 3, 4 and 5). The smaller studies will be used to optimize our strength exercise models, inactivity models and episodic memory tests before commencing with the main experiments. The studies described in appendix 1 test strength exercise and inactivity methods. The studies described in appendix 2 test episodic memory tests.

The three main studies will all make use of the same animal procedures and general set-up, making use of (exercise) interventions, physical fitness tests and cognitive tests. Three different mouse models will be used in this same experimental set-up:
- Aged mice (and young mice as controls) (Appendix 3)
- Human APOE4 mice and human APOE3 mice (appropriate controls) (Appendix 4)
- Amyloid AD model mice (J20 mice) and wild-type controls (Appendix 5)

Animal procedures

The types of animal procedures that will be performed can be divided in three categories, which show the basic components and primary outcome measures of all experiments that are described.

1. Interventions to induce or reduce physical activity, or induce cognitive stimulation:
We are currently characterizing methods which allow us to induce strength exercise and inactivity on a voluntary basis. It could of course be that over the course of the project we have to conclude that these models do not suffice (e.g. aged mice do not engage in our voluntary forms of exercise). If this is the case, we will use forced methods. This is not expected though, since aged mice do still perform voluntary (resistance) running.[1]
- Voluntary models will be based on changing housing conditions. For example; for strength exercise this will be either the placement of a burrowing tube in the cage or access to a running wheel which is loaded to induce resistance while running.[1,2]
- Forced methods will be based on placing the mouse in an apparatus which forces it to be physically active or not. We will use an inclined treadmill to induce strength exercise in the form of resistance running.[3,4,5]

Endurance exercise and cognitive stimulation methods have already been developed and tested by other research groups. Voluntary running in a running wheel or forced running on treadmill will be used as a model for endurance exercise.[4,6] To induce cognitive stimulation we will house mice in enriched environments in which no running wheel will be present, placement of different objects and tubes and re-arrangement of these objects in a large cage will provide cognitive stimulation.[6]

2. Physical fitness tests
These tests will be used to measure the physical effects of the exercise intervention strategies and to confirm the exercise intervention had their predicted effects on musculature and the cardiovascular system.
- Using behavioural tests we will generate data on the functional effects of (in)activity on strength, endurance and coordination. These tests include the grip strength meter test to measure strength, a treadmill running tests to measure endurance and the balance beam test to measure coordination.

3. Cognitive tests:
Several tests will be used to measure the cognitive capabilities and cognitive decline in our mouse models. Many of these are based on voluntary behaviour of the mouse, but positive or negative reinforcers may be used.
- Cognitive tests include tests for episodic memory (The custom build automated Time-Place learning test and the What-Where-When-Which test (WWWW-test)[7], spatial learning (Morris Water Maze) and working memory (Y-maze test). To motivate the mice in the Time-Place learning task we will deprive the mice of food. This novel test is described in more detail in appendix 2.

References:

[1] Soffe, Z., Radley-Crabb, H. G., McMahon, C., Grounds, M. D. & Shavlakadze, T. Effects of loaded voluntary wheel exercise on performance and muscle hypertrophy in young and old male C57Bl/6J mice. *Scand. J. Med. Sci. Sports* 1–17 (2015). doi:10.1111/sms.12416

[2] [redacted]

[3] Sakakima H. *et al.* The Effects of Aging and Treadmill Running on Soleus and Gastrocnemius Muscle Morphology in the Senescence-Accelerated Mouse (SAMP1). *The Journals of Gerontology* **59,** 1015-1021 (2004)

[4] Kemi, O. J., Loennechen, J. P., Wisløff, U. & Ellingsen, Ø. Intensity-controlled treadmill running in mice: cardiac and skeletal muscle hypertrophy. *J. Appl. Physiol.* **93,** 1301–9 (2002)

[5] Aparicio, V. a *et al.* Effects of high-whey-protein intake and resistance training on renal, bone and metabolic parameters in rats. *Br. J. Nutr.* **105,** 836–845 (2011).

[6] van Praag, H., Kempermann, G. & Gage, F. H. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat. Neurosci.* **2,** 266–270 (1999).

[7] Davis, K. E., Easton, A., Eacott, M. J. & Gigg, J. Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *J. Alzheimers. Dis.* **33,** 681–98 (2013).

3.4.3 Beschrijf en benoem de logische samenhang van deze verschillende onderdelen en de eventuele fasering in de uitvoering. Vermeld eventuele mijlpalen en keuzemomenten.

Phasing and Go/NoGo
As was mentioned in section 3.4.1 further testing of our novel strength exercise method, our novel inactivity method and the episodic memory tests is needed to ensure they can be used in the aged and AD-model mice. Only after we successfully tested a strength exercise and inactivity method, and successfully tested the episodic memory test, we can commence with the main experiments that use these methods.

Achievability Go/NoGo
One strength exercise method, resistance running, has been successfully tested in aged mice by another research group.[1] Moreover, with regard to our novel strength exercise method; Burrowing is performed up to at least 12 months of age (unpublished results). Therefore, we are confident that we can use these strength exercise methods in aged mice. Similarly, episodic memory has been tested in aged mice in our research group.[2] One of our episodic memory tasks will be based on object recognition and exploration by mice. A recent study performed in our group successfully used object recognition tasks in ten-month old mice. Other groups have used object recognition tasks to test hAPOE and AD mice of the age at which we will use these mice in our the experiments.[3-5] Therefore we are confident that we can successfully optimize and apply the proposed episodic memory tests (described in appendix 2).
Whether we can induce inactivity using our novel method is unsure: Theoretically mice housed under standard conditions may already be quite inactive, creating a ''floor effect'' which does not allow us to induce further inactivity. If this turns out to be the case, we will move on without the ''inactivity group'' in the experiments proposed in appendix 3,4 and 5.

Coherence
Our objectives will be achieved by testing the different (exercise) interventions and their effects on physical functioning and brain functioning in healthy aged, AD and human APOE4 mice using a similar set-up for all groups (see appendix 3, 4 and 5).

The physical fitness tests serve to confirm the expected effects of our (exercise) interventions on physical functioning of the mice. The cognitive tests serve will be used to investigate whether our interventions influence age-, APOEe4- or AD-related cognitive decline in our three mouse models (3.4.2.). The need to test both physical and cognitive parameters in every experiment emerges from a theoretical point of view: We hypothesize that physical/metabolic fitness and brain health/cognitive function are linked because signalling molecules and growth factors that regulate adaptations in musculature and cardiovascular systems influence brain health as well.[6]

Coherence – Main experiments
The use of the three different mouse models allows us to identify whether (in)activity and/or cognitive stimulation may differentially affects brain health and functioning in healthy elderly, elderly at risk for sporadic AD (APOE4 genotype) and AD patients. Testing these mouse models in the same set-up will help to compare them, and to specify at which phase in the development of AD these types of therapeutic interventions can be most useful. The use of an episodic memory test is expected to reveal cognitive decline in aged, APOEe4 and AD models. Episodic memory performance can serve as an early marker for AD in humans and therefore could be useful to place our different mouse models and the effects of (exercise) interventions at in different phases of AD pathogenesis.[7]

Together with the studies performed by our partners in the consortium, these animal studies should provide a clear starting point to devise

improved/optimized intervention strategies in relevant human groups: elderly at risk for AD, APOEe4 carriers and AD patients.

References:

[1] Soffe, Z., Radley-Crabb, H. G., McMahon, C., Grounds, M. D. & Shavlakadze, T. Effects of loaded voluntary wheel exercise on performance and muscle hypertrophy in young and old male C57Bl/6J mice. *Scand. J. Med. Sci. Sports* 1–17 (2015). doi:10.1111/sms.12416

[2] [redacted]

[3] Bour, A. *et al.* Middle-aged human apoE4 targeted-replacement mice show retention deficits on a wide range of spatial memory tasks. *Behav. Brain Res.* **193,** 174–82 (2008).

[4] Nichol, K. E., Parachikova, A. I. & Cotman, C. W. Three weeks of running wheel exposure improves cognitive performance in the aged Tg2576 mouse. *Brain Res.* **184,** 124–32 (2007).

[5] Harris, J. A. *et al.* Many neuronal and behavioral impairments in transgenic mouse models of Alzheimer's disease are independent of caspase cleavage of the amyloid precursor protein. *J. Neurosci.* **30,** 372–381 (2010).

[6] Raichlen, D. a & Polk, J. D. Linking brains and brawn: exercise and the evolution of human neurobiology. *Proc. Biol. Sci.* **280,** 20122250 (2013).

[7] Dubois, B. *et al.* Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol.* **9,** 1118–1127 (2010).

3.4.4 Benoem de typen dierproeven. Vul per type dierproef een bijlage Beschrijving dierproeven in.	
Volgnummer	Type dierproef
1	Testing of strength exercise methods and physical inactivity methods in mice.
2	Testing of episodic memory tasks in mice
3	Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in adult and aged mice.
4	Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in hAPOE4 mice.
5	Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in Amyloid Alzheimer’s Disease model mice.
6	
7	
8	
9	
10	

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10500
1.2	Provide the name of the licenced establishment.	Rijksuniversiteit Groningen
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 Type of animal procedure: Testing of strength exercise methods and physical inactivity methods in mice.

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.

General strategy
The aim of the animal procedures described below is to test interventions that can be used as 1) a strength exercise method and 2) to induce inactivity in adult mice, aged mice and AD mice. The impact of these interventions will be measured by way of physical fitness tests. After characterizing these methods, we can select one strength exercise method and one inactivity method to use in the studies described in appendices 3-5.

Physical fitness tests will be used to measure functional effects of the physical (in)activity interventions by measuring muscle strength, stamina and motor coordination in a repeated measures design: These tests will be performed before, halfway through and after a 10-12 week intervention. A final physical fitness test, the treadmill endurance test, will only be performed after the intervention period. Additional data is gained by analysing muscle and heart tissue and can be used to confirm that the functional behavioural changes are accompanied by expected adaptations of muscle tissue and the cardiovascular system.

Testing sequence
1. Testing of strength exercise and inactivity methods in adult (male and female) mice.
2. Subsequently, we will test the same models in aged (male and female) C57Bl6 mice.
3. Finally, we will test both methods in (male and female) transgenic AD model mice (J20 mice, described in section B).

Only one suitable strength exercise (out of 3, see below) method and one inactivity (out of 3, see below) method will be tested at one time, going through the sequence of events described above step by step. If we successfully characterize one strength exercise method and one inactivity method, no further testing will be performed to characterize possible other methods.

Testing strength exercise and inactivity methods in both sexes in adult, aged and AD mice is needed to ensure our (in)activity interventions can be successfully applied in the main experiments described in appendices 3, 4 and 5.

Physical fitness tests: Primary outcome parameters and related animal procedures
- Strength will be measured using a grip strength meter. The mice are allowed to hold on to the meter, then the animal is pulled backwards by the tail until it releases the grid. Peak force is the primary outcome parameter to measure strength in the forelimbs or in all four limbs respectively.
- Stamina will be measured using the inverted screen test. Animals are placed on a grid, the grid is turned upside down and placed above a padded surface. The time until the animal has to let go of the grid and falls is taken as a measure for stamina.
- Endurance will be measured by running on a treadmill. At the end of lane on the treadmill there is a shock-grid which delivers a shock of ~0.3mA, which is used as a negative reinforcer to induce running on the treadmill. In a progressively harder endurance test the animals run until exhaustion. Maximum running speed (Vmax) is taken as the primary outcome measure.
- Coordination will be tested in the balance beam test. During this test, the animal is placed on a thin beam or rod, which it has to cross to reach its home-cage. The time needed to cross the beam and the number of errors (paw-slips) will be taken as primary outcome parameters for coordination.

We use these outcome parameters because they allow us to test whether the strength exercise and inactivity interventions have resulted in the expected functional changes.

Tissue analyses: Primary outcome parameters
- Muscles will be weighed to check for muscle hypertrophy (an effect of strength exercise) or muscle wasting (an effect of aging and inactivity).
- Hearth weight will be determined to check for cardiac hypertrophy (an effect of endurance exercise).
- Fat pads will be weighed to check for a decrease or increase in fat mass (an effect of endurance exercise or inactivity respectively).
- Additionally, muscle tissue will be collected and frozen so that it can be analysed using histological techniques. This can be used to check whether the cross sectional diameter of fast-twitch muscle fibres has increased (an effect of strength exercise) or decreased (a possible effect of inactivity).

These outcome parameters are matched to the results from the physical fitness tests.

Selection criteria for the proper strength exercise and inactivity interventions
A strength exercise method will be considered successful if it induces increases grip strength and does not increase endurance to the level of established endurance exercise methods (i.e voluntary running, this data can be found in literature and in our previous studies). Moreover, a good strength exercise method induces muscle hypertrophy or the diameter of fast-twitch fibres in skeletal muscles. An inactivity method will be considered successful if it reduces strength or endurance as compared to home-cage control mice.

[1] [redacted]

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

1. Strength exercise and inactivity interventions

Strength exercise methods
- Burrowing: For five days a week, a burrowing tube filled with sand and pebbles will be placed in the

cage of the mouse at the beginning of the dark phase so that it can empty it by burrowing overnight.
- Pseudo-burrowing: For five days a week, a burrowing tube filled with small wood chips (normal bedding for the mice) will be placed in the cage of the mouse at the beginning of the dark phase so that it can empty it by burrowing overnight. This is not a form of exercise for the mice, as the wood chips are very lightweight and easy to remove.
Pseudo-burrowers are a control group for the burrowing group. Burrowers need larger cages as compared to normally housed mice to make room for the burrowing tube and burrowing substrate that is removed. Moreover, balancing on the burrowing tube may affect physical fitness and coordination. To control for these factors, pseudo-burrowers will be used when the burrowing strength exercise method is selected.
- Resistance-running, low or high intensity, in a custom made set-up: Mice will be housed with a standard running wheel, a resistance on the rotation of the wheel is induced by hanging weights on a pulley attached to the axis of the running wheel.
- Tilted treadmill running: Mice will be placed in a treadmill for five days a week for 30-60 minutes. To mimic strength exercise, the treadmill will be tilted to an angle (~30 degrees) and intensity will be increased. Finally, an established rat weight pulling treadmill running paradigm will be adapted in terms of weight load to work for mice. [1,2]

Inactivity methods
- Inactivity induced by changes in cage size: Mice will be housed in a small cage (1/2 the size of a normal mouse cage).
- Inactivity induced by reduced climbing: Mice will be housed in a normal cage with a plastic lid, in which they cannot climb.
- Inactivity induced by changes in cage size and reduced climbing: Mice will be housed in a normal or small cage (1/2 the size of a normal mouse cage) with a plastic lid, in which the cannot climb.

2. Physical fitness tests
As mentioned in section A, physical fitness tests will be performed before, halfway through and after a 10-12 week (in)activity intervention.

- The grip strength test consists of two sessions: a total of four forelimb measurements and four forelimb and hind limb measurements. Each measurement takes approximately 10 seconds with breaks of 1 minute in between trials and a break of 2,5 minutes between sessions, adding up to a total duration of approximately 15 minutes.
- The inverted screen test starts with a 5 minute habituation period and consists of two trials with a 10 minute break in between trials. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests. Depending on how long the mouse can hold on to the screen, this test may take up to 50 minutes in total.
- Depending on the number of beams and rods that are included, the balance beam test can take 10-30 minutes. Addition of rods and beams that are of different width and shape can improve the ability of the test to discriminate between different exercise regimes. For every beam three trials will be performed, with 30 second breaks between them. Between to different beams, there will be a break of 1,5 minutes. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests.
- The treadmill endurance test consists of one trial. The duration of this trial is dependent on the endurance of the mouse and may take up to 45 minutes in total. The test is performed until exhaustion, which is classified as staying on the shock grid for 10 seconds despite prodding by the researcher. Early sign of fatigue are lowering of the tail and failing to run in the front part of the treadmill. Two days before the actual endurance test, a habituation trial of 12 minutes is performed to reduce novelty stress during the test.

[1] Kemi, O. J., Loennechen, J. P., Wisløff, U. & Ellingsen, Ø. Intensity-controlled treadmill running in mice: cardiac and skeletal muscle hypertrophy. *J. Appl. Physiol.* **93,** 1301–9 (2002)
[2] Aparicio, V. a *et al.* Effects of high-whey-protein intake and resistance training on renal, bone and metabolic parameters in rats. *Br. J. Nutr.* **105,** 836–845 (2011).

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

A mixed repeated measures design is possible for the physical fitness tests, this design yields more power with a smaller number of animals as compared to a one-way ANOVA. A power analyses for a repeated measures ANOVA (between groups, based on a large effect size (f=0.4), an alpha of 0.05, beta of 0.8 and 0.5 correlation between measures) revealed a total of 45 mice is needed to test three groups (n=15 per group):

1. Home cage controls
2a. Strength Exercise
2b. Pseudo strength exercise (if burrowing is strength exercise method)
3. Inactive

We will need an additional group to control for certain elements of the strength exercise method depending on the strength exercise method that is suitable: the addition of pseudo-burrowers when the burrowing is our most promising method). We will adjust our power analyses based observations made (and data collected) in the studies that are being performed currently.[1]

We cannot re-use animals from these pilots since some of our primary outcome parameters depend on taking out and analysing muscles, fat tissue and the hearth.

[1] [redacted]
Unpublished results, [redacted]

B. The animals

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

1. Male and female C57Bl6J mice (mus musculus), bred by a certified European breeding company or bred in our own facilities, both adult (2-3 months old) and aged (14 months), will be used. (A maximum of adult: n=119, aged: n=133)
2. Male and female AD (J20) mice, on a C57Bl6J background, bred in our own facilities, adult (6 months) mice will be used. (n=140)

Testing in both sexes, aged mice and AD mice are needed to ensure our strength exercise and inactivty methods can be successfully applied in the main experiments described in appendices 3, 4 and 5. The AD model mice will be tested at the same age as they will be in the experiments described in appendix 5: 6 months old. At this age, these mice show a decline in several cognitive domains and formation of Amyloid plaques (a hallmark of AD). [1-3]

Estimated numbers
In every testing round, a total number of max. 17 adult, max. 19 aged and max. 20 AD model mice per group is needed to perform an experiment containing the following groups:
1. Home cage controls
2a. Strength Exercise
2b. Pseudo strength exercise (if burrowing is strength exercise method)
3. Inactive

We estimate to test a maximum of two strength and inactivity methods in adult mice, aged mice and in the AD mice. As soon as we successfully characterize one strength exercise method and one inactivity method, no further testing will be performed to characterize possible other methods.

Calculations
- Groups of 15 mice are needed to test under ideal conditions. If the selected strength exercise models are voluntary, an additional 2 mice may need to be added to ensure at least 15 animals perform the desired behaviour (observations made for resistance running and burrowing in our unpublished experiment being analysed currently).
- Due to mortality that comes with the aging process we are likely to lose up to 5% of the animals. An

additional 5% of the mice are expected to show signs of morbidity that come with age and which may confound results via systemic effects on the brain and behavior (e.g. development of stereotypic behavior, seizures, or tumor development).
- We expect to lose 15% of the AD mice (J20 strain) due to increased mortality which is part of the AD phenotype.[3,4]

Therefore we will include max. (15+2=) 17 animals per group when testing in adult mice and (15+2+5% + 5%=) 19 animals per group when testing in aged mice. When testing with AD mice, we will include (15+2+15%=) 20 animals per group.

In our most conservative scenario, in which two rounds of testing are necessary for adult, aged and AD mice, this adds up to:

- 2*(3 groups*17) = 102 adult mice
Or, when Pseudo-Burrowing is taken along for 1 round:
- 3 groups*17 + 4 groups*17 = 119 adult mice

- 2*(3 groups*19) = 114 aged mice
Or, when Pseudo-Burrowing is taken along for 1 round:
- 3 groups*19 + 4 groups*19 = 133 aged mice

- 2*(3 groups*20) = 120 AD mice
Or, when Pseudo-Burrowing is taken along for 1 round:
- 3 groups*20 + 4 groups*20 = 140 AD mice

References

[1] Mucke, L. et al. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050–4058 (2000).
[2] Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A. & Van Eldik, L. J. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front. Genet.* **5**, 1–23 (2014).
[3] https://www.jax.org/strain/006293 (Website of Jackson, the supplier of this mouse strain)
[4] Cheng, I. H., Scearce-Levie, K., Legleiter, J., Palop, J. J., Gerstein, H., Bien-Ly, N., … Mucke, L. (2007). Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. *J Biol Chem*, **282**(**33**), 23818–23828. http://doi.org/10.1074/jbc.M701078200

C. Re-use

Will the animals be re-used?

No, continue with question D.

X Yes > Explain why re-use is considered acceptable for this animal procedure.

If the anticipated behaviour in the strength exercise and inactivity intervention is clearly not performed, we will cancel that round of pilots prematurely and re-use the animals in a next pilot round after a sufficient wash-out period of two weeks. This will reduce the number of animals needed to perform these studies.

The test that will produce the most adverse effects, treadmill running, will only be used at the end of a study and thus will not have been performed if a pilot is cancelled prematurely. The other physical fitness tests are classified as *mild* and can be used multiple times in the same animal. As a consequence we expect that the animals that would be re-used to do not experience an excessive amount of discomfort and results in the next round of pilots are not confounded.

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

X 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: These experiments cannot be performed without the use of animals. The tests require the use of complex models as they have to mimic the interventions that could be performed in humans and there are no other models available that can be used to mimic the complex physiological adaptations in body (and brain) that are the consequence of physical (in)activity.

Reduction: Within each testing round, we cannot reduce the number of animals any further due potential loss of statistical power. By testing the most promising methods one-at-the-time in one-group-at-a-time (adult, aged, AD mice) we can stop and move on to another method as soon as we successfully characterized a model in the desired test group. This reduces the number of animals that are needed as compared to the situation in which all models are tested at once (or in all groups at once).

Refinement: Refinement: We aim to develop (exercise) interventions based on voluntary behaviour. This reduces stress due to handling as compared to forced methods. Habituation is used in behavioural tests where protocol allows for it. Furthermore, the shock intensity used in the treadmill test has been optimized in a previously performed pilot experiment and is set as low as possible (while still being a strong enough stimulus).

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

The behavioural tests will include habituation phases to reduce stress by handling or exposure to the test apparatus. Furthermore, the animals are habituated to handling and being moved from room to room during a habituation period at the start of each experiment.

Repetition and duplication

E. Repetition

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

A literature search was used to identify existing exercise methods and their effects on the brain. We found that the effects of strength exercise or induced inactivity have scarcely been tested in rodents and that there are no truly well-characterized (voluntary) strength exercise methods available for mice at this moment.

Accommodation and care

F. Accommodation and care

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

No

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

The animals will be housed individually during the 12 weeks of (exercise) intervention because it is necessary to monitor the physical activity of the individual. (e.g. amount burrowed, climbed or run)

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.

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

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

Animals may experience mild pain during the treadmill running test due to the mild electrical shock which is used as a negative reinforcer. This pain cannot be relieved, this would compromise the treadmill endurance test.

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

I. Other aspects compromising the welfare of the animals

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

Animals may be mildly stressed by the treadmill exercise and by our novel method of induced inactivity. They may be moderately stressed by the treadmill endurance test.

Explain why these effects may emerge.

We expect no adverse effects on animal welfare during any of the voluntary exercise interventions, as these interventions are based on enriching the environment of the animal. With regard to the inactivity models, we may expect that housing in a smaller cage or a cage without climbing possibilities will adversely affect the welfare of the animal as it cannot engage in natural forms of locomotor activity. We expect treadmill running to induce mild/moderate stress and discomfort induced by the shock grid at the end of the treadmill lanes and moderate discomfort due to running until exhaustion.

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

The adverse welfare effects of the inactivity model cannot be avoided as this is an intrinsic part of the method. However, we will carefully monitor the inactivity groups to ensure they do not exert stereotypical behaviour that may compromise their well-being. It is of note that our pilot experiments did not reveal that this temporary increase in inactivity causes abnormal overt behaviour.

A habituation phase can assure the animals get used to running in the treadmill at a low pace, reducing stress and discomfort to some extent. We cannot replace the foot shock as it is essential to motivate the animals. (Animals will habituate to and eventually ignore touching and poking by the researcher)
Careful monitoring of the animals during the treadmill endurance test ensures they are placed back in their home cage as soon as they are exhausted.

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.

An animal will be euthanized if a significant decline in general health status arises, indicated by for example a matt and lustreless fur, or by showing signs of pain. Mice will be euthanized if they lose more than 15% bodyweight, show piloerection or show abnormal behaviour, like strong decline in feeding/drinking, and reluctance to accept handling. We do not expect the animals to reach such a condition as a consequence of the experiments, but adverse health effects may arise sporadically during the aging process of the mice.

Indicate the likely incidence.

5% in aged animals. None of these cases are to be expected in the adult mice.

In the AD model mice (J20 mice) we might expect some morbidity related to the 15% increase in mortality, but the cause of premature death in this line is unknown (so we cannot be certain about increased morbidity).[1]

References:
[1] Cheng, I. H., Scearce-Levie, K., Legleiter, J., Palop, J. J., Gerstein, H., Bien-Ly, N., … Mucke, L. (2007). Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. *J Biol Chem*, **282**(**33**), 23818–23828. http://doi.org/10.1074/jbc.M701078200

K. Classification of severity of procedures

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

All of the behavioural tasks, the inactivity models and the possible forced treadmill running as a strength exercise model result in mild discomfort.

The treadmill endurance tests results in moderate discomfort due to the use of exhaustion as an endpoint is the test.

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.

To collect muscle tissue, fat pads and the hearth, which are needed to confirm the physiological effects of the (in)activity intervention.

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'.	10500
1.2	Provide the name of the licenced establishment.	Rijksuniversiteit Groningen
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 2 — Type of animal procedure: Testing of episodic memory tasks in mice

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.

General strategy
The aim of the animal procedures described below is to test two episodic memory tasks that can be used to test different forms of episodic learning and memory in adult mice, aged mice and mouse models of AD:
- The What-where-when-which occasion test (WWWWtest) [1]
- The Automated Time-Place learning test [2]

The tasks cannot be tested in the same groups of mice, or two times in a row, as episodic learning induced by the tasks confounds the results obtained in subsequent episodic learning tests.[3] Therefore, separate groups of mice will be subjected to the two episodic learning tasks in two separate experiments.

Groups of adult, aged and AD mice will be undergoing the episodic memory tasks as described below. If mice learn to perform the tasks successfully we can use the tasks in the experiments described in appendices 3, 4 and 5. If the tasks are not performed successfully, some modifications to the protocol or materials may solve any problems. In the latter case, another round of testing is warranted to ensure the modifications work.

Testing sequence
1. Testing of episodic memory tasks in adult (male and female) C57Bl6 mice.
2. Subsequently, we will test the same tasks in aged (male and female) C57Bl6 mice.
3. Finally, we will test both tasks in (male and female) transgenic AD model mice (J20 mice, described in section B).

Testing in both sexes in adult, aged mice and AD mice, is needed to ensure these episodic memory tasks can be successfully applied in the main experiments described in appendices 3, 4 and 5.

What-Where-When-Which occasion test (WWWWtest) to test episodic cognitive functioning
This task has been described in literature[1] and is based on associative and sequential learning and recognition of novel objects and places. Because the test is quite complex, it is important to optimize the described protocols to make sure that the test can be performed successfully in our facility and by using our materials (i.e objects). The primary outcome parameters for this test are successful discrimination of novel objects, novel locations of familiar objects and association of a certain context and familiar or novel objects.

Test criteria:
If the healthy adult and aged mice tested can successfully discriminate between novel objects, novel locations of familiar objects and association of a certain context and familiar or novel objects the test is considered a success. If the AD mice show a decreased ability to discriminate, but a similar level of exploratory behaviour as compared to the healthy mice, we still consider the test a success: Episodic memory is likely to be affected in early stages of AD and in AD mouse models. We are interested in investigating the effects of our exercise interventions on this form of cognitive decline.

Automated Time-Place learning task to test circadian system dependent episodic cognitive functioning
The automated Time-Place learning test will be used to test time-place learning. This type of learning depends on a form of learning and memory which does not rely on a temporal order of events (as the WWWWtest does) but on the actual circadian time of day on which an event takes place.[4] This is a different form of episodic memory then that which is necessary in the object based tasks described above. We will use a modified home-cage in which the food deprived animal is able to move around freely. In the upper part of the cage three platforms are located, which may or may not give access to a food reward depending on the time of day. The mouse will fall to the bottom of the cage when attempting to cross them at the wrong time of day. The percentage of correct choices is the main output parameter.

Test criteria:
If the healthy adult and aged mice tested can successfully learn to find food at the right time at the right locations the test is considered a success, this is indicated by a percentage of correct choices above chance level. If the AD mice show a decreased ability to learn, but a similar or higher level of attempts to get to the food behaviour as compared to the healthy mice, we still consider the test a success (As was mentioned in the test criteria section of the WWWWtest above: Episodic memory is likely to be affected in early stages of AD and in AD mouse models, we are interested in investigating the effects of our exercise interventions on this form of cognitive decline.

References:
[1] Davis, K. E., Easton, A., Eacott, M. J. & Gigg, J. Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *J. Alzheimers. Dis.* **33,** 681–98 (2013).
[2] Unpublished results and personal communication with [redacted]
[3] [redacted]
[4] [redacted]

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

WWWWtest
During this test the animal is placed in a testing arena containing objects. We will use object based tasks based on the animals' natural tendency to explore novel objects and objects moved to novel locations

(spatial novelty) to measure simple associative forms of memory as well as more complex forms of spatial learning and episodic memory. If the animal remembers the identity of the object or the location of the individual objects it will preferentially explore any novel or moved objects during a test session which is performed after an initial conditioning test session. If the animal formed a poor memory during conditioning (i.e. does not remember which objects were shown in the conditioning session) it will explore all objects to a similar extend. Thus, exploration levels during the test session is primary readout for these tasks. We will extend the often used Novel-Object recognition task and Spatial Location Recognition task by including a more elaborate What-Where-Which occasion task and a What-Where-When task. These tests dependent on the association of an object, location, temporal order of events and contextual information and the integration of these elements to form an episodic memory of an event.

Duration and frequency:
The different object recognition tasks will be tested over the course of 25 days, including 5 days of habituation.[1] These days include several habituation days, acquisition days and testing days. On each day, the animal will spend a total 10 minutes in the testing arena.

Automated Time-Place learning task
The manual Time-Place learning paradigm has been developed by research of our group, and is a successful but labour-intensive and time-consuming procedure.[2] Moreover, the test uses mild electric shocks to stimulate learning. It is impossible to perform this test with large groups of mice due to practical constrains (the time needed to perform the test). However, an automated version of the test has been developed.

We aim to optimize this automated time-place learning protocol so that we can use it for the experiments described in appendices 3, 4 and 5. The animals will need to be food deprived to perform the automated time-place learning task as the time-place learning depends on a food reward which serves a positive reinforcer. We will use a modified home-cage in which the food deprived animal is able to move around freely. In the upper part of the cage three platforms are located, which may or may not give access to a food reward depending on the time of day. The mouse will fall softly to the bottom of the cage (containing soft bedding) when attempting to cross them at the wrong time of day.

Duration and frequency:
The manual version of the time place learning test takes up to 21 days. We may assume the automated version will take as long, but this will need to be tested.

References:
[1] Davis, K. E., Easton, A., Eacott, M. J. & Gigg, J. Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *J. Alzheimers. Dis.* **33,** 681–98 (2013).
[2] [redacted]

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

A repeated measures design is possible for the automated Time-Place learning test: To track progress over the course of 10-21 days a power analyses (within subjects, based on a large effect size (f=0.4), an alpha of 0.05, beta of 0.8 and 0.25 correlation between measures) revealed a total of 10 mice are needed to reveal a learning effect over time for this test.

A one-sample (one-tailed) t-test will be used to determine whether object discrimination differs from chance in the WWWWtest. A power analyses (within subjects, based on a medium-large effect size (d=0.65), an alpha of 0.05, beta of 0.8) revealed a total of 12 mice are needed to reveal a learning effect for this test.

These power analyses will be adjusted based on the results obtained in the first round of experiments.

In order to perform the episodic memory tasks, we need experimentally naïve animals. We cannot re-use animals in these pilots if an episodic memory tasks is performed, since episodic learning affects

subsequent episodic learning.

B. The animals

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

1. Male and female C57Bl6J mice (mus musculus), bred by a certified European breeding company (adult) or bred in our own facilities (aged), both adult (2-3 months old) and aged (14 months), will be used. (adult: n=44, aged: n=50)
2. Male and female Amyloid AD (J20) mice, on a C57Bl6J background, bred in our own facilities, adult (6 months) mice will be used. (n=52)

Testing in both sexes, aged mice and AD mice is needed to ensure our episodic learning tasks can be successfully applied in the main experiments described in appendices 3, 4 and 5. The Amyloid AD model mice will be tested at the same age as they will be in the experiments described in appendix 5: 6 months old. At this age, these mice show a decline in several cognitive domains and formation of Amyloid plaques (a hallmark of AD). [1-3]

A total number of max. 10 adult, 11 aged or 12 AD model mice are needed to test the automated Time-Place learning test once. A total number of 12 adult, 14 aged or 14 AD model mice animals are needed to test the WWWWtest once.

We expect to need at two test rounds for both the WWWWtest the automated Time-Place learning task in adult mice and aged and Amyloid AD mice. But we will stop testing in any of the adult, aged or AD groups as soon as a test is deemed successful.

Calculations
- Groups of 10 and 12 mice are needed to test the automated Time-Place learning task and the WWWW task respectively.
- Due to mortality that comes with the aging process we are likely to lose up to 5% of the animals. An additional 5% of the mice are expected to show signs of morbidity that come with age and which may confound results via systemic effects on the brain and behavior (e.g. development of stereotypic behavior, seizures, or tumor development).
- We expect to lose 15% of the AD mice (J20 strain) due to increased mortality which is part of the AD phenotype.[3,4]

Therefore we will include 10 or 12 animals per group when testing adult mice, (10+5%+5%=) 11 or (12+5%+5%=) 14 animals per group when testing aged mice and (10+15%=) 12 or (12+15%=) 14 animals when testing in AD mice.

In our most conservative scenario, in which two rounds of testing are necessary for adult, aged and AD mice, this adds up to:

- 2*(1 group*10) + 2*(1 group*12) = 44 adult mice
- 2*(1 group*11) + 2*(1 group*14) = 50 aged mice
- 2*(1 group*12) + 2*(1 group*14) = 52 AD mice

References

[1] Mucke, L. et al. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050–4058 (2000).

[2] Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A. & Van Eldik, L. J. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front. Genet.* **5**, 1–23 (2014).

[3] https://www.jax.org/strain/006293 (Website of Jackson, the supplier of this mouse strain)

[4] Cheng, I. H., Scearce-Levie, K., Legleiter, J., Palop, J. J., Gerstein, H., Bien-Ly, N., … Mucke, L. (2007). Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. *J Biol Chem*, **282**(**33**), 23818–23828. http://doi.org/10.1074/jbc.M701078200

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'?

X 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: These experiments cannot be performed without the use of animals. These tests require the use of subjects that are able to learn complex tasks as they have to mimic the complex learning that occurs in humans, this process cannot be modeled in silico or in vitro.

Reduction: Within each testing round, we cannot reduce the number of animals any further due potential loss of power. We will of course stop as soon as we successfully characterized it in the desired test in every group. By first optimizing the tests in adult animals first, we will make it easier to eventually get them working in old animals while using a lower number of animals as compared to the situation in which we would test in aged animals from the start.

Refinement: The automated time-place learning paradigm is a refined version of the manually Time-Place learning paradigm as it does not require the shocks that were used in the manual version. The object recognition tests as well as the automated time-place learning test will include habituation phases to reduce stress by handling or exposure to the test arena or the modified home-cage.

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

The object recognition tests as well as the automated time-place learning test will include habituation phases to reduce stress by handling or exposure to the test arena or the modified home-cage. Furthermore, the animals are habituated to handling during a habituation period at the start of each experiment.

We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

Repetition and duplication

E. Repetition

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

The automated time-place learning apparatus has been developed tested once on technical functioning by our research group. Use of this automated paradigm is a novelty. An extensive literature search revealed that no such an apparatus or voluntary based test paradigm is available.

Repetition: The WWWWtest has been described and succesfully used by other research groups (Davis et al., Journal of Alzheimer's Disease (2013)), but the test is quite complex. To guarantee succesfull implication of the test when we start testing with large numbers of animals in the experiments described in appendices 3, 4 and 5, it is important to optimize the described protocols to make sure that the test can be performed successfully in our facility and using our materials and objects.

Accommodation and care

F. Accommodation and care

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

No

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

While the animals are housed in the Time Place learning apparatus, they are housed individually so that their behavior can be monitred using a camera system and infra-red sensors.

G. Location where the animals procedures are performed

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

X No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort / humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

X No > Continue with question I.

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

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

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

I. Other aspects compromising the welfare of the animals

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

Animals that are used in the Time-Place learning paradigm will lose weight and may experience mild stress and discomfort due to the food restriction or falling from of one of the platforms.

Explain why these effects may emerge.

Food deprivation that is used to motivate the mice in the automated time-place learning task will induce adverse effects on the animals welfare. Furthermore, animal may experience mild stress after they make an error and the platform topples over and they fall down over a small distance.

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

The adverse welfare effects due to food restriction and falling during the automated Time-Place learning task cannot be avoided as this is an intrinsic part of the task and a food reward an essential positive reinforcer. We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

A habituation phase is present in both episodic memory tasks. This reduces stress due to handling and exposure to the tests and test arena.

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.

An animal will be euthanized if a significant decline in general health status arises, indicated by for example a matt and lusterless fur, or by showing signs of pain. Mice will be euthanized if they lose more than 15% bodyweight, show piloerection or show abnormal behavior, like strong decline feeding/drinking,and reluctance to accept handling. We do not expect the animals to reach such a condition as a consequence of the experiments, but adverse health effects may arise sporadically during the aging process of the mice.

Indicate the likely incidence.

5% in aged animals. None of these cases are to be expected in the adult mice.

In the AD model mice (J20 mice) we might expect some morbidity related to the 15% increase in mortality, but the cause of premature death in this line is unknown (so we cannot be certain about increased morbidity).[1]

K. Classification of severity of procedures

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

In both episodic memory tests, due to either novelty stress or handling (only in the WWWWtest), we expect mild discomfort. We expect the discomfort level due to food deprivation to be mild as well.

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.

Animals cannot be re-used for (episodic) learning and memory or behavioral studies if the task is performed succesfully, as they need to be experimentally naïve in order to perform these tasks. Furthermore, brain tissue of the Amyloid AD model mice will be used to confirm AD pathology.

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'.	10500
1.2	Provide the name of the licenced establishment.	Rijksuniversiteit Groningen
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3 Type of animal procedure: Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in adult and aged mice.

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.

Disclaimer: Apart from the general design, the animal procedures and behavioural tests described here have mostly been described in the same manner before (appendix 1 & 2). Novel test described in the following part are the Morris Water Maze and the Y-maze alternation task, their description and justification is found in the final part of the following section. These paragraphs that discuss tests have been marked by an underlined term for your convenience.

General design

Adult (2-3 months) and aged (14 months) mice will undergo an intervention to induce or reduce physical activity in a specific manner (A change of their home-cage size and/or use of a special lid to reduce activity, or a strength exercise or endurance exercise procedure, see appendix 1), or to induce cognitive stimulation (housing in an enriched environment without a running wheel) or both (combined intervention). These (exercise) interventions will form the different groups that will be compared in each experiment. The interventions will last for 10-12 weeks.

Physical fitness tests will be used to assess muscle strength, stamina, endurance and motor coordination before and after the intervention. (see appendix 1) Similarly, a number of behavioural tests will be used to measure cognitive functioning in various domains including spatial learning, working memory and episodic memory after the intervention. (see appendix 2)

Different cognitive test batteries and physical fitness tests, or no tests, will be used in three separate experimental set-ups (see figure 1):

Experimental set-up 1 - The WWWWtest, Y-maze alteration task and Morris-Water-Maze can be performed in one cognitive test battery on the same animals. These tests are combined with physical fitness tests.
Experimental set-up 2 - For the automated Time-Place learning task we need the animals to be naïve with regard to previous episodic learning tasks like the WWWWtest. Moreover, food deprivation is needed in the Time-Place learning task, but food deprivation would be a confounding factor for the other cognitive tasks. This tests is combined with physical fitness tests which are performed before food deprivation.
Experimental set-up 3 - Groups of mice will receive the same interventions (strength exercise, endurance exercise, inactivity, cognitive stimulation), without the cognition or physical fitness testing. These mice will be sacrificed and their brain tissue, blood, cerebrospinal fluid and muscles will be analysed in the lab to determine how (in)activity interventions affect a number of neuro-molecular output parameters.

Growth factor signalling, tissue analyses and neuro-molecular output parameters (experiment 3):
Primary outcome parameters that will be investigated in the brain include growth factors such as Brain Derived Neurotrophic Factor (BDNF), Vascular Endothelial Growth Factor (VEGF) and Insulin-Like Growth Factor 1 (IGF-1) as well as markers for angiogenesis, synaptic plasticity and neurogenesis: These signalling molecules and neuro-molecular processes are known to be pivotal for the effect of endurance exercise on the brain.

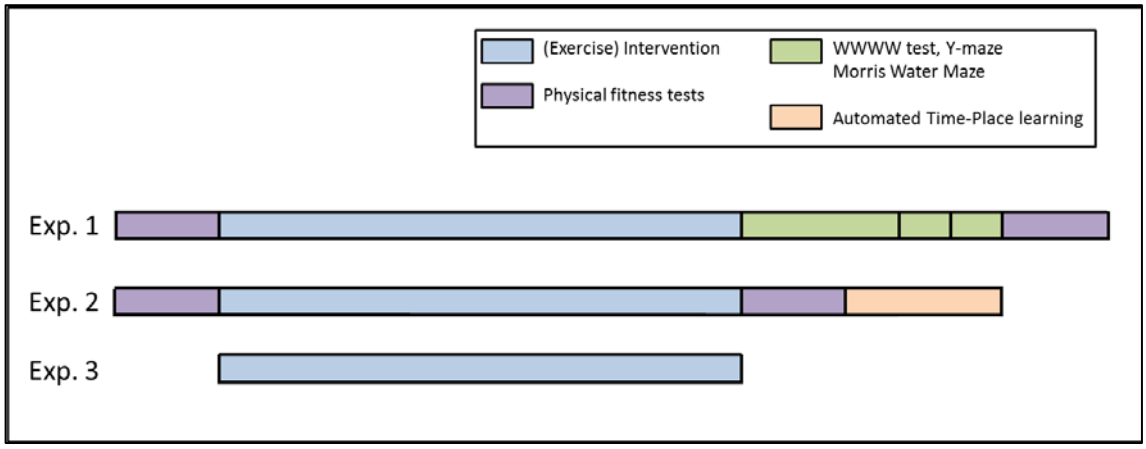


Figure 1. Schematic overview of the 3 experimental set-ups that make use of different (or no) behavioural test batteries.

Primary outcome parameters and related animal procedures: Physical fitness tests (see appendix 1)
The physical fitness tests can be performed subsequent to the cognitive tests. We will investigate whether there are any correlations between physical fitness parameters and cognitive performance.

- Muscle strength will be measured using a grip strength meter. The mice are allowed to hold on to the meter, then the animal is pulled backwards until it releases the grid. Peak force is the primary outcome parameter to measure strength in the forelimbs or in all four limbs respectively.
- Stamina will be measured using the inverted screen test. Animals are placed on a grid, the grid is turned upside down and placed above a padded surface. The time until the animal has to let go of the grid and falls is taken as a measure for stamina.
- Endurance will be measured by running on a treadmill. At the end of lane on the treadmill there is a shock-grid which delivers a shock of ~0.3mA, which is used as a negative reinforce to induce running on the treadmill. In a progressively harder exercise test the animals run until exhaustion. Maximum running speed (Vmax) is taken as the primary outcome measure.
- Motor coordination will be tested in the balance beam test. During this test, the animal is placed on a thin beam or rod, which it has to cross to reach its home-cage. The time needed to cross the beam and

the number of errors (paw-slips) will be taken as primary outcome parameters for coordination.

We use these outcome parameters because they allow us to test whether the (in)activity interventions have resulted in the expected changes in physical functioning in various domains, which will be affected differently by the different interventions.

Primary outcome parameters and related animal procedures: Cognitive tests
We will investigate the effects of the different interventions on different cognitive domains using learning and memory tasks that dependent on different learning and memory systems.

- WWWWtest. *(see appendix 2)* This task has been described in detail in literature[1] and is based on associative and sequential learning and recognition of novel objects and places. The primary outcome parameters for this test are successful discrimination of novel objects, novel locations of familiar objects and association of a certain context and familiar or novel objects.
- The automated Time-Place learning task. *(see appendix 2)* This task will be used to test time-place learning. This type of learning depends on a form of learning and memory which relies on the circadian system.[2] We will use a modified home-cage in which the food deprived animal is able to move around freely. In the upper part of the cage three platforms are located, which may or may not give access to a food reward depending on the time of day. The mouse will fall softly to the bottom of the cage when attempting to cross them at the wrong time of day. The percentage of correct choices is the main output parameter.

- The Morris Water Maze will be used to measure complex forms of spatial learning and memory. Mice are placed in a circular pool and learn to locate a hidden platform. The latency to find the platform as well as the path length to reach the platform will drop over the course of the acquisition trials. This is the primary outcome measure for acquisition of spatial memory. After 48 hours, the mice are placed back in the pool without a platform. The time spent by the mouse in the quadrant where the platform used to be located is the primary outcome measure for retention of spatial memory.
- Working memory will be tested in a Y-maze alternation task. The animal will be placed in a Y-maze, containing three identical arms separated at 120 degree angles from each other. When an animal has intact working memory it will remember which arm it has visited recently. It will explore the three arms more or less subsequently, visiting all three arms approximately the same number of times. The order of the arm exploration is an outcome parameter used to measure working memory.

Justification cognitive tests:
- Episodic memory has been shown to be affected by aging and AD in humans, and in mouse models of AD.[1,3] It is unknown whether exercise interventions can ameliorate these effects. The inclusion of both the object recognition based episodic memory tasks and the Time-Place learning task (dependent on the circadian system) is warranted because both test different forms of episodic memory, which rely on different memory systems. Giving the fast decline in circadian functioning that comes with age, it is important to include circadian system dependent episodic memory tasks.[4]
- These various learning and memory tests allows us to investigate how different memory systems are affected by exercise interventions, inactivity and cognitive stimulation. Performance in the simple object recognition tasks and the Morris Water Maze has been previously shown to be positively affected by voluntary running and an enriched environment and negatively affected by aging. It will therefore be of interest to see whether other forms of physical activity may affect the same learning and memory systems.

References:
[1] Davis, K. E., Easton, A., Eacott, M. J. & Gigg, J. Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *J. Alzheimers. Dis.* **33,** 681–98 (2013).
[2] [redacted]
[3] Dubois, B. *et al.* Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol.* **9,** 1118–1127 (2010).
[4] Kondratova, A. a & Kondratov, R. V. The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci.* **13**, 325–35 (2012).

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

Disclaimer: The animal procedures and behavioural tests described here have mostly been described before (appendix 1 & 2). Novel test described in the following part are the Morris Water Maze and the Y-maze alteration task, their description and justification is found in the final part of the following section. These paragraphs that discuss these tests have been marked by an underlined term for your convenience.

(Exercise) Intervention methods
One of the inactivity methods and one of the strength exercise methods described in appendix 1 will be selected. Depending on whether the inactivity and strength methods are voluntary or forced, voluntary running or treadmill running will be used to serve as a model for endurance exercise.

Cognitive stimulation will be induced using an enriched environment. An enriched environment will consist of a larger cage filled with a number of objects and maze-like parts that are arranged differently every week. This environment will not contain a running wheel in the cognitive stimulation intervention, but will contain a running wheel in the combined intervention.[2]

All of these methods will be implemented for a period of 10-12 weeks before cognitive testing will commence.

Duration and frequency of physical fitness tests (See appendix 1)
- The grip strength test consists of two sessions: a total of four forelimb measurements and four forelimb & hind limb measurements. Each measurement takes approximately 10 seconds with breaks of 1 minute in between trials and a break of 2,5 minutes between sessions, adding up to a total duration of approximately 15 minutes.
- The inverted screen test starts with a 5 minute habituation period and consists of two trials with a 10 minute break in between trials. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests. Depending on how long the mouse can hold on to the screen, this test may take up to 50 minutes in total.
- Depending on the number of beams and rods that are included, the balance beam test can take 10-30 minutes. Addition of rods and beams that are of different width and shape can improve the ability of the test to discriminate between different exercise regimes. For every beam three trials will be performed, with 30 second breaks between them. Between two different beams, there will be a break of 1,5 minutes. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests.
- The treadmill endurance test consists of one trial. The duration of this trial is dependent on the endurance of the mouse and may take up to 45 minutes in total. The test is performed until exhaustion, which is classified as staying on the shock grid for 10 seconds despite prodding by the researcher. Early signs of fatigue are lowering of the tail and failing to run in the front part of the treadmill. Two days before the actual endurance test, a habituation trial of 12 minutes is performed to reduce novelty stress during the test.

Duration and frequency of cognitive tests (description of the tests is given in the part above)
- The different object recognition tasks will be tested over the course of 25 days, including 5 days of habituation.[1] These days include several habituation days, acquisition days and testing days. On each day, the animal will spend a total 10 minutes in the testing arena.
- The manual version of the time place learning test takes up to 21 days. We may assume the automated version will take as long, but this will need to be tested.
- In the Morris water maze an animal will be subjected to 4 trials (of maximum 8-10 minutes) per day, over the course of 5-7 days (until the animals has learned the task). Each trial will be finished when the mouse has located the platform and a trial. Probe tests will take place 48 hours after the last acquisition day, and will take 8 minutes per animal.
- The Y-maze alternation task takes a total of 8 minutes per animal.

Justification of selected approaches
The 10-12 weeks (exercise) intervention period is chosen based on literature and results from a first series of studies we performed.[1] Apart from the automated Time-Place learning task, the cognitive tests that are being performed have a standardized design which has been shown to give optimal results by our group and others. The protocol of the automated Time-Place learning task will be optimized using the results obtained from the experiments described in appendix 2. We will shorten the duration of this task as much as possible.

References:
[1] [redacted]
Unpublished results, [redacted]
[2] van Praag, H., Kempermann, G. & Gage, F. H. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat. Neurosci.* **2,** 266–270 (1999).

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

Behavioural testing experimental set-up (1 & 2)
A mixed repeated measures design is possible for the physical fitness tests, but not for the cognitive tests (the performance of the same test twice poses a confounding factor because learning performing the same cognitive tasks in the same setting will become easier every time a cognitive test is repeated). A power analyses for a one-way ANOVA (based on a large effect size (f=0.4), an alpha of 0.05 and beta of 0.8) revealed a total of 80 mice is needed to test five groups (n=16 per group) and a total of 73 animals is needed to test four groups (n=19 per group, rounded up). The groups that are needed are listed in section B.

Because many of the interventions and the automated Time-Place learning test have not been used before, we cannot determine the exact expected effect sizes from literature (the above effect size is an educated guess based on voluntary running studies). We will adjust our power analyses based on effect sizes and expected variation in primary outcome parameters based on studies that are being performed currently and the studies described in appendices 1 and 2.

Tissue analysis experimental set-up (3)
A power analyses for a one-way ANOVA (based on a larger effect size (f=0.5), an alpha of 0.05 and beta of 0.8) revealed a total of 53 mice is needed to test five groups (n=11 per group, rounded up) and a total of 48 animals is needed to test four groups (n=12 per group). The groups that are needed are listed in section B.

Pseudo-burrowers
We will need an additional group to control for certain elements of the strength exercise method depending on the strength exercise method that is suitable: the addition of pseudo-burrowers when the burrowing is our most promising method. We will adjust our power analyses based observations made (and data collected) in the studies that are being performed currently.[1]

[1] [redacted]
Unpublished results, [redacted]

B. The animals

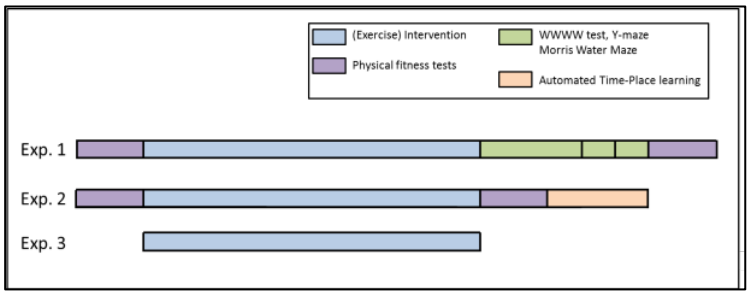
Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

1. Male and female C57Bl6J mice (mus musculus), bred by a certified European breeding company or bred in our own facilities, both adult (2-3 months old) and old (14 months) mice will be used. A maximum of n=518 adult and n=578 aged mice will be used (most conservative estimates calculated below).

Estimated numbers

Groups (A) - To investigate the effects of physical inactivity and various exercise regimes, groups of max. 18 adult or 20 aged mice are needed:
1. Home cage controls
2a. Strength Exercise
2b. Pseudo strength exercise (if burrowing is strength exercise method)
3. Inactive
4. Endurance exercise (voluntary or treadmill running)
5. Endurance exercise + strength exercise

Groups (B) - To investigate differential effects of cognitive stimulation, aerobic exercise and a combination of both, groups of max. 21 adult or 23 aged mice are needed:
1. Home-cage control
2. Enriched environment
3. Endurance exercise (voluntary running)
4. Endurance exercise + enriched environment (combined intervention)



Experimental set-up 1, 2 and 3 (see scheme on the right and section A) will be performed with groups A and B. So in total six experiments will be performed.

Because of practical limitations, we cannot test more than six of these groups in one experimental set-up. We need to perform three separate experiments: One to test for the effects of these interventions on Time-Place learning, one to test the effects in the other cognitive domains, one without behavioral tests for tissue collection. (see the first part of section A).

Calculations
- Groups of 16 or 19 mice are needed to test cognition of 5 or 4 groups under ideal conditions. If the selected exercise methods are voluntary, an additional 2 mice may need to be added to ensure at least 16 or 19 mice perform the desired behaviour (Based on our observations made for (resistance) running and burrowing).
- Due to mortality that comes with the aging process we are likely to lose up to 5% of the animals. An additional 5% of the mice are expected to show signs of morbidity that come with age and which may confound results via systemic effects on the brain and behavior (e.g. development of stereotypic behavior, seizures, or tumor development).

Therefore we will include max (16+2=) 18 or (19+2=) 21 animals per group when behavioral testing adult mice and (16+2+5%+5%=) 20 or (19+2+5%+5%=) 23 animals per group when behavioral testing in aged mice.

- Finally, for both adult and aged mice groups of mice that will not be tested in any of the cognitive tests will be included to collect brain tissue for immuno-histochemical analyses. (experimental set-up 3) These group sizes are smaller, but subject to the same alterations as the groups that are behaviourally tested: Addition of 2 animals for some voluntary methods and 5% mortality and 5% morbidity in aged animals.

Therefore we will include max (11+2=) 13 or (12+2=) 14 animals per group when testing adult mice or (11+2+5%+5%) 15 or (12+2+5%+5%=)16 animals per group when testing in aged mice.

In our most conservative scenario, which will be adjusted to include fewer animals wherever possible, this adds up to:

Groups (A) experimental set-up 1 + 2
- 5 groups*18 = 90 adult mice per experiment (=180)
Or, when Pseudo-Burrowing is added:

Groups (A) experimental set-up 3:
- 5 groups*13 = 65 adult mice
Or, when Pseudo-Burrowing is added:

- 6 groups*18 = 108 adult mice per experiment (=216) And: - 5 groups*20 = 100 aged mice per experiment (=200) Or, when Pseudo-Burrowing is added: - 6 groups*20 = 120 aged mice per experiment (=240)	- 6 groups*13 = 78 adult mice And: - 5 groups*15 = 75 aged mice Or, when Pseudo-Burrowing is added: - 6 groups*15 = 90 aged mice
Groups (B) experimental set-up 1 + 2 - 4 groups*21 = 84 adult mice per experiment (=168) And - 4 groups*23 = 92 aged mice per experiment (=184)	Groups (B) experimental set-up 3: - 4 groups*14 = 56 adult mice - 4 groups*16 = 64 aged mice

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'?

X 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: These experiments cannot be performed without the use of animals. The experiments require the use of complex animal models as they have to mimic the effects that the interventions may have in humans: There are no other models available that can be used to mimic the complex physiological adaptations in body (and brain) that are the consequence of cognitive and physical (in)activity. Moreover, no known *in silico* or *in vitro* models are available to mimic complex learning and memory as is measured by the various cognitive tasks.

Reduction: We cannot reduce the number of animals any further due potential loss of power. We will minimize the number of animals needed as much as possible by optimizing the tests that are used as described in appendices 1 and 2. We will adjust our power analyses and therefore the group size according to the results of those studies.

Refinement: Refinement: We aim to develop (exercise) interventions based on voluntary behaviour. This reduces stress due to handling as compared to forced methods. Habituation is used in behavioural tests. Furthermore, the shock intensity used in the treadmill test has been optimized in a previously performed pilot experiment and is set as low as possible (while still being a strong enough stimulus). The automated time-place learning paradigm is a refined version of the manually Time-Place learning paradigm: It does not require shocks as negative reinforcers, as was the case in the manual version.

Depending on the results of experiments described in Appendix 1, we may be able to characterize the effects of our interventions and exercise regimes mainly based on tissue analyses rather than behavioural testing (e.g. muscle weight instead of the grip strength test). We will need to confirm that this is possible in these studies, but if it is possible we will reduce the number of physical fitness tests as much as possible to further minimize discomfort (without compromising our read-out of physical fitness).

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

The behavioural tests will include habituation phases to reduce stress by handling animals or exposure to the test arena or the modified home-cage. Furthermore, all animals are habituated to handling and the

behavioural test room before the start of behavioural testing.

We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

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.

A literature search was used to identify existing exercise methods and their effects on the brain. We found that the effects of strength exercise or induced inactivity have scarcely been tested in rodents and that there are no well-characterized (voluntary) strength exercise methods available for mice at this moment. The effects of exposure to an enriched environment and the effects of endurance exercise on spatial memory have been tested before in both adult and aged mice. However, we need to include these groups in order to make a direct comparison between these existing interventions and our novel and combined interventions. Moreover, the effects of running or exposure to an enriched environment on episodic memory have not been investigated in detail, and not at all with respect to circadian Time-Place Association.

Accommodation and care

F. Accommodation and care

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

☐ No

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

The animals will be housed individually during the (exercise) intervention because it is necessary to monitor the physical activity of the individual. (e.g. amount burrowed or distance run)

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?

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

Animals may experience mild pain during the treadmill running test due to the mild electrical shock which is used as a negative reinforcer. This pain cannot be relieved, this would compromise the treadmill endurance test.

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

I. Other aspects compromising the welfare of the animals

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

Animals may be mildly stressed by the treadmill exercise and by our novel method of induced inactivity. They may be moderately stressed by the treadmill endurance test. During other behavioural tests animals may experience discomfort/novelty stress induced by handling and exposure to test arena's or apparatus. Moreover, animals that are used in the Time-Place learning paradigm will lose weight and may experience mild stress or discomfort due to the food restriction or falling from the platforms on a soft surface.

Explain why these effects may emerge.

We expect no adverse effects on animal welfare during any of the voluntary exercise interventions, as these interventions are based on enriching the environment of the animal.

With regard to the inactivity method, we may expect that the housing in a smaller cage or a cage without climbing possibilities will adversely affect the welfare of the animal to some extend as it cannot fully engage in natural forms of locomotor activity.

We expect treadmill running to induce mild stress induced by the shock grid at the end of the treadmill lanes. The treadmill endurance test may exert moderate discomfort because of running until exhaustion combined with the use of shocks.

Animals experience novelty stress when exposed to an unfamiliar environment, this is likely to occur in any of the test arena's we use. In the Morris Water Maze, some additional discomfort arises due to the forced swimming that is part of the maze protocol (mice are not particularly fond of swimming).

Finally, the food deprivation that is used to motivate the mice in the automated time-place learning task will induce adverse effects on the animals welfare due to a reduced caloric intake, weight loss and hunger.

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

The possible adverse welfare effects of the inactivity method cannot be avoided as this is an intrinsic part of the method. However, we will carefully monitor the inactivity groups to ensure they do not exert stereotypical behaviour (already when testing the method as indicated in appendix 1, but also during the experiments described in the current appendix). It is of note that our pilot experiments did not reveal that this temporary increase in inactivity causes abnormal overt behaviour.

The adverse welfare effects due to food restriction and falling during the automated Time-Place learning task cannot be avoided as this is an intrinsic part of the task and a food reward an essential positive reinforcer. We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

A habituation phase is present in all our behavioural tests whenever it is possible, including the Morris Water Maze. This reduces stress due to handling and exposure to the tests.

A habituation phase can assure the animals get used to running in the treadmill at a low pace, reducing stress and discomfort to some extent. We cannot replace the foot shock as it is essential to motivate the animals. (Animals will habituate to and eventually ignore prodding by the researcher, for example.) Careful monitoring of the animals during the treadmill endurance test ensures they are placed back in their home cage as soon as they are exhausted.

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.

An animal will be euthanized if a significant decline in general health status arises, indicated by for example a matt and lustreless fur, or by showing signs of pain. Mice will be euthanized if they lose more than 15% bodyweight, show piloerection or show abnormal behaviour, like a strong decline feeding/drinking, and reluctance to accept handling. We do not expect the animals to reach such a condition as a consequence of the experiments, but adverse health effects may arise sporadically during the aging process of the mice.

Indicate the likely incidence.

5% in the aged groups. None of these cases are to be expected in the adult mice.

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').

Any of the behavioural tasks apart from the Time-Place learning test, because of novelty stress and the use of a mild shock in the treadmill test: 'Mild'. The automated time-place learning test, because of the food deprivation: 'Mild'

The treadmill endurance tests results in 'Moderate' discomfort due to the use of exhaustion as an endpoint in the test.

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.

All animals will be killed to collect muscle tissue, fat pads and the hearth, which are needed to confirm the physiological effects of the (in)activity intervention.

The animals that undergo experimental set-up 3 (no behavioral testing) need to be killed in order to take out brain tissue for detailed (immuno-histochemical) analyses.

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'.	10500
1.2	Provide the name of the licenced establishment.	Rijksuniversiteit Groningen
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 4 Type of animal procedure: Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in hAPOE4 mice.

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.

Disclaimer: The animal procedures and behavioural tests described here have been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use human APOE mice. The paragraphs that discuss additional output parameters and set-up that will be used have been marked by an underlined term for your convenience.

General design

Aged female (14 months) transgenic human APOEe4 and human APOEe3 mice will be used. This age was chosen because a clear cognitive phenotype emerges at 14 months in female hAPOE4 mice.[1] We will investigate whether (exercise) interventions influence this phenotype in terms of functional changes (learning and memory) as well as changes on a neuro-molecular level. hAPOEe3 mice will serve as controls for the hAPOEe4 mice because murine APOE is not comparable in function and structure to the ''neutral '' hAPOEe3 in humans. Clear effects of APOE4 genotype on (age-related) cognitive decline are found in female, but not male mice.[1] Similarly, in humans the increased risk of AD in APOE4 carriers seems restricted to females.[2] Hence, we will test in female mice only.

These mice will undergo an intervention to induce or reduce physical activity in a specific manner (A change of their home-cage size and/or use of a special lid to reduce activity, or a strength exercise or endurance exercise procedure, see appendix 1) or to induce cognitive stimulation (housing in an enriched environment without a running wheel) or both (combined intervention). These (exercise) interventions will form the different groups that will be compared in each experiment. The interventions will last for

10-12 weeks.

Physical fitness tests will be used to assess muscle strength, stamina, endurance and motor coordination before and after the intervention. (see appendix 1) Similarly, a number of behavioural tests will be used to measure cognitive functioning in various domains including spatial learning, working memory and episodic memory after the intervention. (see appendix 2)

Different cognitive test batteries and physical fitness tests, or no tests, will be used in three separate experimental set-ups (see figure 1):

Experimental set-up 1 - The WWWWtest, Y-maze alteration task and Morris-Water-Maze can be performed in one cognitive test battery on the same animals. These tests are combined with physical fitness tests.
Experimental set-up 2 - For the automated Time-Place learning task we need the animals to be naïve with regard to previous episodic learning tasks like the WWWWtest. Moreover, food deprivation is needed in the Time-Place learning task, but food deprivation would be a confounding factor for the other cognitive tasks. This tests is combined with physical fitness tests which are performed before food deprivation.
Experimental set-up 3 - Groups of mice will receive the same interventions (strength exercise, endurance exercise, inactivity, cognitive stimulation), without the cognition or physical fitness testing. These mice will be sacrificed and their brain tissue, blood, cerebrospinal fluid and muscles will be analysed in the lab to determine how (in)activity interventions affect a number of neuro-molecular output parameters.

Growth factor signalling, tissue analyses and neuro-molecular output parameters (experiment 3):
Primary outcome parameters that will be investigated in the brain include growth factors such as Brain Derived Neurotrophic Factor (BDNF), Vascular Endothelial Growth Factor (VEGF) and Insulin-Like Growth Factor 1 (IGF-1) as well as markers for angiogenesis, synaptic plasticity and neurogenesis: These signalling molecules and neuro-molecular processes are known to be pivotal for the effect of endurance exercise on the brain.

In addition, specific markers for the APOEe4 phenotype will be investigated: These include ApoE receptors found in the brain and on the blood-brain barrier, lipoprotein vesicle concentrations in the serum and atherosclerotic plaques found in the vasculature.

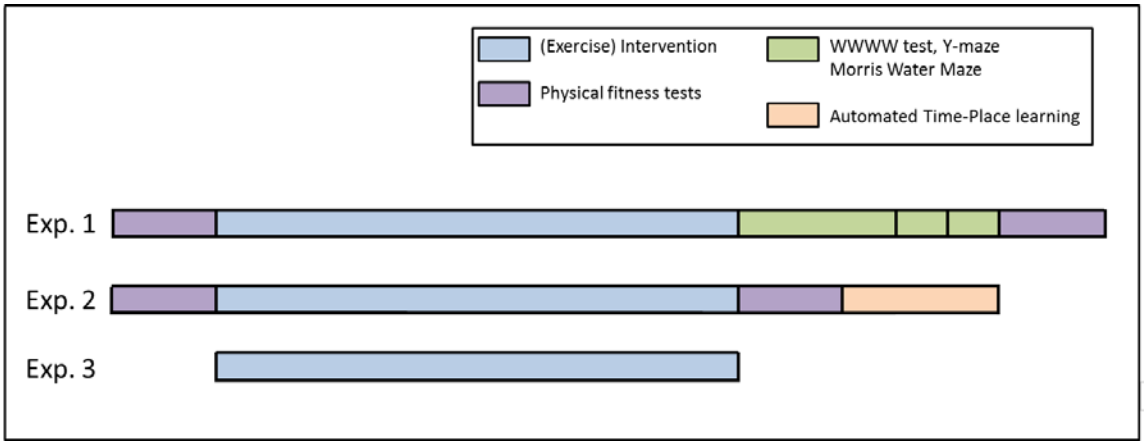


Figure 1. Schematic overview of the 3 experimental set-ups that make use of different (or no) behavioural test batteries.

Primary outcome parameters and related animal procedures: Physical fitness tests (see appendix 1)
The physical fitness tests can be performed subsequent to the cognitive tests. We will investigate whether there are any correlations between physical fitness parameters and cognitive performance.

- Muscle strength will be measured using a grip strength meter. The mice are allowed to hold on to the

meter, then the animal is pulled backwards until it releases the grid. Peak force is the primary outcome parameter to measure strength in the forelimbs or in all four limbs respectively.
- Stamina will be measured using the inverted screen test. Animals are placed on a grid, the grid is turned upside down and placed above a padded surface. The time until the animal has to let go of the grid and falls is taken as a measure for stamina.
- Endurance will be measured by running on a treadmill. At the end of lane on the treadmill there is a shock-grid which delivers a shock of ~0.3mA, which is used as a negative reinforce to induce running on the treadmill. In a progressively harder exercise test the animals run until exhaustion. Maximum running speed (Vmax) is taken as the primary outcome measure.
- Motor coordination will be tested in the balance beam test. During this test, the animal is placed on a thin beam or rod, which it has to cross to reach its home-cage. The time needed to cross the beam and the number of errors (paw-slips) will be taken as primary outcome parameters for coordination.

We use these outcome parameters because they allow us to test whether the (in)activity interventions have resulted in the expected changes in physical functioning in various domains, which will be affected differently by the different interventions.

Primary outcome parameters and related animal procedures: Cognitive tests
We will investigate the effects of the different interventions on different cognitive domains using learning and memory tasks that dependent on different learning and memory systems.

- WWWWtest. *(see appendix 2)* This task has been described in detail in literature[1] and is based on associative and sequential learning and recognition of novel objects and places. The primary outcome parameters for this test are successful discrimination of novel objects, novel locations of familiar objects and association of a certain context and familiar or novel objects.
- The automated Time-Place learning task. *(see appendix 2)* This task will be used to test time-place learning. This type of learning depends on a form of learning and memory which relies on the circadian system.[2] We will use a modified home-cage in which the food deprived animal is able to move around freely. In the upper part of the cage three platforms are located, which may or may not give access to a food reward depending on the time of day. The mouse will fall softly to the bottom of the cage when attempting to cross them at the wrong time of day. The percentage of correct choices is the main output parameter.

- The Morris Water Maze will be used to measure complex forms of spatial learning and memory. Mice are placed in a circular pool and learn to locate a hidden platform. The latency to find the platform as well as the path length to reach the platform will drop over the course of the acquisition trials. This is the primary outcome measure for acquisition of spatial memory. After 48 hours, the mice are placed back in the pool without a platform. The time spent by the mouse in the quadrant where the platform used to be located is the primary outcome measure for retention of spatial memory.
- Working memory will be tested in a Y-maze alternation task. The animal will be placed in a Y-maze, containing three identical arms separated at 120 degree angles from each other. When an animal has intact working memory it will remember which arm it has visited recently. It will explore the three arms more or less subsequently, visiting all three arms approximately the same number of times. The order of the arm exploration is an outcome parameter used to measure working memory.

Justification cognitive tests:
- Episodic memory has been shown to be affected by aging and AD in humans, and in mouse models of AD.[1,3] It is unknown whether exercise interventions can ameliorate these effects. The inclusion of both the object recognition based episodic memory tasks and the Time-Place learning task (dependent on the circadian system) is warranted because both test different forms of episodic memory, which rely on different memory systems. Giving the fast decline in circadian functioning that comes with age, it is important to include circadian system dependent episodic memory tasks.[4]
- These various learning and memory tests allows us to investigate how different memory systems are affected by exercise interventions, inactivity and cognitive stimulation. Performance in the simple object recognition tasks and the Morris Water Maze has been previously shown to be positively affected by voluntary running and an enriched environment and negatively affected by APOEe4 genotype in mice. It

will therefore be of interest to see whether other forms of physical activity may affect the same learning and memory systems.

References:

[1] Davis, K. E., Easton, A., Eacott, M. J. & Gigg, J. Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *J. Alzheimers. Dis.* **33,** 681–98 (2013).

[2] [redacted]

[3] Dubois, B. *et al.* Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol.* **9,** 1118–1127 (2010).

[4] Kondratova, A. a & Kondratov, R. V. The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci.* **13**, 325–35 (2012).

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

Disclaimer: The animal procedures and behavioural tests described here have been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use human APOE mice.

(Exercise) Intervention methods

One of the inactivity methods and one of the strength exercise methods described in appendix 1 will be selected. Depending on whether the inactivity and strength methods are voluntary or forced, voluntary running or treadmill running will be used to serve as a model for endurance exercise.

Cognitive stimulation will be induced using an enriched environment. An enriched environment will consist of a larger cage filled with a number of objects and maze-like parts that are arranged differently every week. This environment will not contain a running wheel in the cognitive stimulation intervention, but will contain a running wheel in the combined intervention.[2]

All of these methods will be implemented for a period of 10-12 weeks before cognitive testing will commence.

Duration and frequency of physical fitness tests (As described in appendix 1)

- The grip strength test consists of two sessions: a total of four forelimb measurements and four forelimb & hind limb measurements. Each measurement takes approximately 10 seconds with breaks of 1 minute in between trials and a break of 2,5 minutes between sessions, adding up to a total duration of approximately 15 minutes.
- The inverted screen test starts with a 5 minute habituation period and consists of two trials with a 10 minute break in between trials. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests. Depending on how long the mouse can hold on to the screen, this test may take up to 50 minutes in total.
- Depending on the number of beams and rods that are included, the balance beam test can take 10-30 minutes. Addition of rods and beams that are of different width and shape can improve the ability of the test to discriminate between different exercise regimes. For every beam three trials will be performed, with 30 second breaks between them. Between two different beams, there will be a break of 1,5 minutes. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests.
- The treadmill endurance test consists of one trial. The duration of this trial is dependent on the endurance of the mouse and may take up to 45 minutes in total. The test is performed until exhaustion, which is classified as staying on the shock grid for 10 seconds despite prodding by the researcher. Early signs of fatigue are lowering of the tail and failing to run in the front part of the treadmill. Two days before the actual endurance test, a habituation trial of 12 minutes is performed to reduce novelty stress during the test.

Duration and frequency of cognitive tests (description of the tests is given in the part above)

- The different object recognition tasks will be tested over the course of 25 days, including 5 days of habituation.[1] These days include several habituation days, acquisition days and testing days. On each day, the animal will spend a total 10 minutes in the testing arena.

- The manual version of the time place learning test takes up to 21 days. We may assume the automated version will take as long, but this will need to be tested.
- In the Morris water maze an animal will be subjected to 4 trials (of maximum 8-10 minutes) per day, over the course of 5-7 days (until the animals has learned the task). Each trial will be finished when the mouse has located the platform and a trial. Probe tests will take place 48 hours after the last acquisition day, and will take 8 minutes per animal.
- The Y-maze alternation task takes a total of 8 minutes per animal.

Justification of selected approaches
The 10-12 weeks (exercise) intervention period is chosen based on literature and results from a first series of studies we performed.[1] Apart from the automated Time-Place learning task, the cognitive tests that are being performed have a standardized design which has been shown to give optimal results by our group and others. The protocol of the automated Time-Place learning task will be optimized using the results obtained from the experiments described in appendix 2. We will shorten the duration of this task as much as possible.

References:
[1] [redacted]
Unpublished results, [redacted]

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

Disclaimer: The animal procedures and behavioural tests described here have been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use human APOE mice. Additional information is underlined for your convenience.

A mixed repeated measures design is possible for the physical fitness tests, but not for the cognitive tests (the performance of the same test twice poses a confounding factor because learning performing the same cognitive tasks in the same setting will become easier every time a cognitive test is repeated). A power analyses for a one-way ANOVA (based on a large effect size (f=0.4), an alpha of 0.05 and beta of 0.8) revealed a total of 80 mice is needed to test five groups (n=16 per group) and a total of 73 animals is needed to test four groups (n=19 per group, rounded up). This power analysis takes into account that we wish to investigate a possible interaction between genotype (APOEe3 or APOEe4) and (exercise) intervention type. The groups that are needed are listed in section B.

Because many of the interventions and the automated Time-Place learning test have not been used before, we cannot determine the exact expected effect sizes from literature (the above effect size is an educated guess based on voluntary running studies and the few studies performed with APOEe4 mice). We will adjust our power analyses based on effect sizes and expected variation in primary outcome parameters based on studies that are being performed currently and the studies described in appendices 1 and 2.

Tissue analysis experimental set-up (3)
A power analyses for a one-way ANOVA (based on a larger effect size (f=0.5), an alpha of 0.05 and beta of 0.8) revealed a total of 53 mice is needed to test five groups (n=11 per group, rounded up) and a total of 48 animals is needed to test four groups (n=12 per group). The groups that are needed are listed in section B.

Pseudo-burrowers
We will need an additional group to control for certain elements of the strength exercise method depending on the strength exercise method that is suitable: the addition of pseudo-burrowers when the burrowing is our most promising method. We will adjust our power analyses based observations made (and data collected) in the studies that are being performed currently.[1]

References:

[1] [redacted]

Unpublished results, [redacted]

B. The animals

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

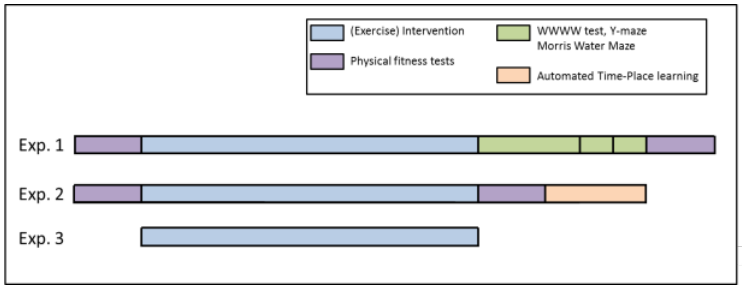
1. Female human APOEe4 and human APOEe3 mice[1,2] (C57Bl6J background, mus musculus), bred in our own facilities, 14 months old mice will be used. A maximum of n=578 hAPOEe4 and n=578 hAPOEe3 mice (most conservative estimates calculated below).

Disclaimer: Due to the similarity in set-up, calculations shown below are the similar compared to those shown in appendix 3. The two changes is that only aged mice are used in the currently described experiments and that we use hAPOE mice , additional information about these mice is underlined for your convenience.

Estimated numbers

Groups (A) - To investigate the effects of physical inactivity and various exercise regimes, groups of max. 20 APOEe3 or APOEe4 mice are needed.
1. Home cage controls
2a. Strength Exercise
2b. Pseudo strength exercise (if burrowing is strength exercise method)
3. Inactive
4. Endurance exercise (voluntary or treadmill running)
5. Endurance exercise + strength exercise

Groups (B) - To investigate differential effects of cognitive stimulation, aerobic exercise and a combination of both, groups of max. 23 hAPOEe3 or hAPOEe4 mice are needed:
1. Home-cage control
2. Enriched environment
3. Endurance exercise (voluntary running)
4. Endurance exercise + enriched environment (combined intervention)



Experimental set-up 1, 2 and 3 (see scheme on the right and section A) will be performed with groups A and B. So in total six experiments will be performed.

Because of practical limitations, we cannot test more than six of these groups in one experimental set-up. We need to perform three separate experiments: One to test for the effects of these interventions on Time-Place learning, one to test the effects in the other cognitive domains, one without behavioral tests for tissue collection. (see the first part of section A).

Calculations

- Groups of 16 or 19 wild-type mice are needed to test cognition of 5 or 4 groups under ideal conditions. If the selected exercise methods are voluntary, an additional 2 mice may need to be added to ensure at least 16 or 19 mice perform the desired behaviour (Based on our observations made for (resistance) running and burrowing).
- Due to mortality that comes with the aging process we are likely to lose up to 5% of the hAPOEe3 and hAPOEe4 mice. An additional 5% of the mice are expected to show signs of morbidity that come with and which may confound results via systemic effects on the brain and behavior (e.g. development of stereotypic behavior, seizures, or tumor development).

Therefore we will include max (16+2+5%+5%=) 20 or (19+2+5%+5%=) 23 animals per group when

behavioral testing in hAPOEe3 and hAPOEe4 mice.

- Finally, for both hAPOEe3 and hAPOEe4 mice groups of mice that will not be tested in any of the cognitive tests will be included to collect brain tissue for immuno-histochemical analyses. (experimental set-up 3) These group sizes are smaller, but subject to the same alterations as the groups that are behaviourally tested: Addition of 2 mice for some voluntary methods and 5% mortality and 5% morbidity in aged hAPOEe3 and hAPOEe4 mice.

Therefore we will include max (11+2+5%+5%) 15 or (12+2+5%+5%=)16 animals per group when testing in aged hAPOEe3 and hAPOEe4 mice.

In our most conservative scenario, which will be adjusted to include fewer animals wherever possible, this adds up to:

Groups (A) experimental set-up 1 + 2
- 5 groups*20 = 100 APOEe4 mice per experiment (=200)
Or, when Pseudo-Burrowing is added:
- 6 groups*20 = 120 APOEe4 mice per experiment (=240)
And:
- 5 groups*20 = 100 APOEe3 mice per experiment (=200)
Or, when Pseudo-Burrowing is added:
- 6 groups*20 = 120 APOEe3 mice per experiment (=240)

Groups (A) experimental set-up 3:
- 5 groups*15 = 75 APOEe4 mice
Or, when Pseudo-Burrowing is added:
- 6 groups*15 = 90 APOEe4 mice
And:
- 5 groups*15 = 75 APOEe3 mice
Or, when Pseudo-Burrowing is added:
- 6 groups*15 = 90 APOEe3 mice

Groups (B) experimental set-up 1 + 2
- 4 groups*23 = 92 APOEe4 mice per experiment (=184)
And
- 4 groups*23 = 92 APOEe3 mice per experiment (=184)

Groups (B) experimental set-up 3:
- 4 groups*16 = 64 APOEe4 mice
- 4 groups*16 = 64 APOEe3 mice

References:
[1] Knouff, C. *et al.* Apo E structure determines VLDL clearance and atherosclerosis risk in mice. *J. Clin. Invest.* **103,** 1579–86 (1999).
[2] Sullivan, P. M. *et al.* Targeted Replacement of the Mouse Apolipoprotein E Gene with the Common Human APOE3 Allele Enhances Diet-induced Hypercholesterolemia and Atherosclerosis. *J. Biol. Chem.* **272,** 17972–17980 (1997).

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'?

x 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.

Disclaimer: The information provided in section D has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use an human APOE mice. Additional information is underlined for your convenience.

Replacement: These experiments cannot be performed without the use of animals. The experiments require the use of complex animal models as they have to mimic the effects that the interventions may have in humans: There are no other models available that can be used to mimic the complex physiological adaptations in body (and brain) that are the consequence of cognitive and physical

(in)activity. Moreover, no known *in silico* or *in vitro* models are available to mimic complex learning and memory as is measured by the various cognitive tasks. Finally, the interaction between APOE genotype and (exercise) interventions can only be observed *in vivo* as this depends on complex physiological mechanisms with are poorly understood.

Reduction: We cannot reduce the number of animals any further due potential loss of power. We will minimize the number of animals needed as much as possible by optimizing the tests that are used as described in appendices 1 and 2. We will adjust our power analyses and therefore the group size according to the results of those studies.

Refinement: Refinement: We aim to develop (exercise) interventions based on voluntary behaviour. This reduces stress due to handling as compared to forced methods. Habituation is used in behavioural tests. Furthermore, the shock intensity used in the treadmill test has been optimized in a previously performed pilot experiment and is set as low as possible (while still being a strong enough stimulus). The automated time-place learning paradigm is a refined version of the manually Time-Place learning paradigm: It does not require shocks as negative reinforcers, as was the case in the manual version.

Depending on the results of experiments described in Appendix 1, we may be able to characterize the effects of our interventions and exercise regimes mainly based on tissue analyses rather than behavioural testing (e.g. muscle weight instead of the grip strength test). We will need to confirm that this is possible in these studies, but if it is possible we will reduce the number of physical fitness tests as much as possible to further minimize discomfort (without compromising our read-out of physical fitness).

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

The behavioural tests will include habituation phases to reduce stress by handling animals or exposure to the test arena or the modified home-cage. Furthermore, all animals are habituated to handling and the behavioural test room before the start of behavioural testing.

We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

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.

Disclaimer: The information provided in section E has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use an human APOE mice. Additional information is underlined for your convenience.

A literature search was used to identify existing exercise methods and their effects on the AD-like phenotype. We found that the effects of strength exercise or induced inactivity have scarcely been tested in rodents and that there are no well-characterized (voluntary) strength exercise methods available for mice at this moment. The effects of exposure to an enriched environment and the effects of endurance exercise on spatial memory and APOEe4 related cognitive decline has been tested only three times in APOEe4 mouse models.[1-3] Mixed results from these studies warrant further investigation and testing using additional learning and memory tests. Moreover, we need to include these groups in order to make a direct comparison between these existing interventions and our novel and combined interventions.

To date, no studies have investigated the effect of (endurance) exercise on episodic memory in hAPOEe4 mice. In fact, the testing of Time-Place association or another form of episodic memory has not been attempted in this hAPOE4 mice at all.

References:
[1] Nichol, K., Deeny, S. P., Seif, J., Camaclang, K. & Cotman, C. W. Exercise improves cognition and hippocampal plasticity in APOE epsilon4 mice. *Alzheimers. Dement.* **5,** 287–94 (2009).
[2] ApoE4 impairs hippocampal plasticity isoform-specifically and blocks the environmental stimulation of synaptogenesis and memory. **13,** 273–282 (2003).
[3] Environmental enrichment stimulates neurogenesis in apolipoprotein E3 and neuronal apoptosis in apolipoprotein E4 transgenic mice. *J. Neurochem.* **100,** 202–10 (2007).

Accommodation and care

F. Accommodation and care

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

☐ No

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

The animals will be housed individually during the (exercise) intervention because it is necessary to monitor the physical activity of the individual. (e.g. amount burrowed or distance run)

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?

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

Animals may experience mild pain during the treadmill running test due to the mild electrical shock which is used as a negative reinforcer. This pain cannot be relieved, this would compromise the treadmill endurance test.

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

I. Other aspects compromising the welfare of the animals

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

Disclaimer: The information provided in section I has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use hAPOE mice.

Animals may be mildly stressed by the treadmill exercise and by our novel method of induced inactivity. They may be moderately stressed by the treadmill endurance test. During other behavioural tests animals may experience discomfort/novelty stress induced by handling and exposure to test arena's or apparatus. Moreover, animals that are used in the Time-Place learning paradigm will lose weight and

may experience mild stress or discomfort due to the food restriction or falling from the platforms on a soft surface.

Explain why these effects may emerge.

We expect no adverse effects on animal welfare during any of the voluntary exercise interventions, as these interventions are based on enriching the environment of the animal.

With regard to the inactivity methods, we may expect that the housing in a smaller cage or a cage without climbing possibilities will adversely affect the welfare of the animal to some extend as it cannot fully engage in natural forms of locomotor activity.

We expect treadmill running to induce mild stress induced by the shock grid at the end of the treadmill lanes. The treadmill endurance test may exert moderate discomfort because of running until exhaustion combined with the use of shocks.

Animals experience novelty stress when exposed to an unfamiliar environment, this is likely to occur in any of the test arena's we use. In the Morris Water Maze, some additional discomfort arises due to the forced swimming that is part of the maze protocol (mice are not particularly fond of swimming).

Finally, the food deprivation that is used to motivate the mice in the automated time-place learning task will induce adverse effects on the animals welfare due to a reduced caloric intake, weight loss and hunger.

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

The possible adverse welfare effects of the inactivity method cannot be avoided as this is an intrinsic part of the method. However, we will carefully monitor the inactivity groups to ensure they do not exert stereotypical behaviour (already when testing the method as indicated in appendix 1, but also during the experiments described in the current appendix). It is of note that our pilot experiments did not reveal that this temporary increase in inactivity causes abnormal overt behaviour.

The adverse welfare effects due to food restriction and falling during the automated Time-Place learning task cannot be avoided as this is an intrinsic part of the task and a food reward an essential positive reinforcer. We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

A habituation phase is present in all our behavioural tests whenever it is possible, including the Morris Water Maze. This reduces stress due to handling and exposure to the tests.

A habituation phase can assure the animals get used to running in the treadmill at a low pace, reducing stress and discomfort to some extent. We cannot replace the foot shock as it is essential to motivate the animals. (Animals will habituate to and eventually ignore prodding by the researcher, for example.) Careful monitoring of the animals during the treadmill endurance test ensures they are placed back in their home cage as soon as they are exhausted.

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.

Disclaimer: The information provided in section J has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use an hAPOE mice.

An animal will be euthanized if a significant decline in general health status arises, indicated by for example a matt and lustreless fur, or by showing signs of pain. Mice will be euthanized if they lose more than 15% bodyweight, show piloerection or show abnormal behaviour, like a strong decline

feeding/drinking, and reluctance to accept handling. We do not expect the animals to reach such a condition as a consequence of the experiments, but adverse health effects may arise sporadically during the aging process of the mice.

Indicate the likely incidence.

5% in aged mice, which are all mice described in this appendix.

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').

Any of the behavioural tasks apart from the Time-Place learning test, because of novelty stress and the use of a mild shock in the treadmill test: 'Mild'. The automated time-place learning test, because of the food deprivation: 'Mild'

The treadmill endurance tests results in 'Moderate' discomfort due to the use of exhaustion as an endpoint in the test.

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.

All animals will be killed to collect muscle tissue, fat pads and the hearth, which are needed to confirm the physiological effects of the (in)activity intervention. Serum and tissue will be collected to confirm the animals have developed hAPOE4 phenotype (lipoprotein concentrations and possibly atherosclerotic lesions).

The animals that undergo experimental set-up 3 (no behavioral testing) need to be killed in order to take out brain tissue for detailed (immuno-histochemical) analyses.

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'.	10500
1.2	Provide the name of the licenced establishment.	Rijksuniversiteit Groningen
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 5 Type of animal procedure: Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in Amyloid Alzheimer's Disease model mice.

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.

Disclaimer: The animal procedures and behavioural tests described here have been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use J20 AD-model mice. The paragraphs that discuss additional output parameters and set-up that will be used have been marked by an underlined term for your convenience.

General design

Amyloid Alzheimer's disease model mice, J20 mice (6 months old) will be used. This transgenic model is mimics Amyloid plaque formation which is a pathological hallmark of AD. For further details on this specific strain see section B. This age was chosen because a clear cognitive phenotype and AD pathological hallmarks have just emerged at 6 months.[1] We will investigate whether (exercise) interventions influence the progression of the AD-like phenotype in terms of functional changes (learning and memory) and pathological hallmarks (plaque formation). Wild-type littermates will serve as controls for the J20 AD-model mice.

These mice will undergo an intervention to induce or reduce physical activity in a specific manner (A change of their home-cage size and/or use of a special lid to reduce activity, or a strength exercise or endurance exercise procedure, see appendix 1), or to induce cognitive stimulation (housing in an enriched environment without a running wheel) or both (combined intervention). These (exercise) interventions will form the different groups that will be compared in each experiment. The interventions will last for 10-12 weeks.

Physical fitness tests will be used to assess muscle strength, stamina, endurance and motor coordination before and after the intervention. (see appendix 1) Similarly, a number of behavioural tests will be used to measure cognitive functioning in various domains including spatial learning, working memory and episodic memory after the intervention. (see appendix 2)

Different cognitive test batteries and physical fitness tests, or no tests, will be used in three separate experimental set-ups (see figure 1):

Experimental set-up 1 - The WWWWtest, Y-maze alteration task and Morris-Water-Maze can be performed in one cognitive test battery on the same animals. These tests are combined with physical fitness tests.
Experimental set-up 2 - For the automated Time-Place learning task we need the animals to be naïve with regard to previous episodic learning tasks like the WWWWtest. Moreover, food deprivation is needed in the Time-Place learning task, but food deprivation would be a confounding factor for the other cognitive tasks. This tests is combined with physical fitness tests which are performed before food deprivation.
Experimental set-up 3 - Groups of mice will receive the same interventions (strength exercise, endurance exercise, inactivity, cognitive stimulation), without the cognition or physical fitness testing. These mice will be sacrificed and their brain tissue, blood, cerebrospinal fluid and muscles will be analysed in the lab to determine how (in)activity interventions affect a number of neuro-molecular output parameters.

Growth factor signalling, tissue analyses and neuro-molecular output parameters (experiment 3):
Primary outcome parameters that will be investigated in the brain include growth factors such as Brain Derived Neurotrophic Factor (BDNF), Vascular Endothelial Growth Factor (VEGF) and Insulin-Like Growth Factor 1 (IGF-1) as well as markers for angiogenesis, synaptic plasticity and neurogenesis: These signalling molecules and neuro-molecular processes are known to be pivotal for the effect of endurance exercise on the brain.

In addition, specific markers for AD-like pathology will be investigated. These include Amyloid plaque formation detected by immunohistochemistry and $A\beta_{1-42}$ levels in the cerebrospinal fluid and brain tissue detected by western blot.

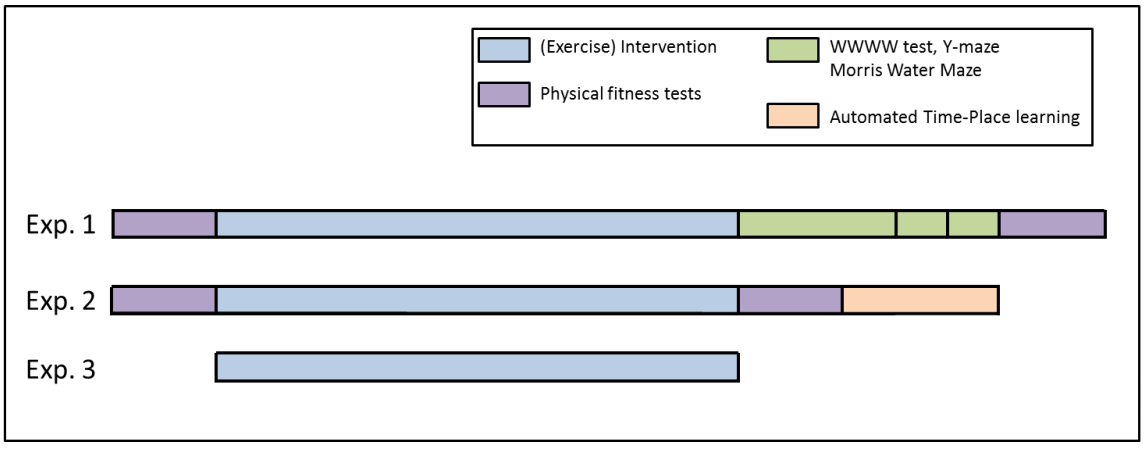


Figure 1. Schematic overview of the 3 experimental set-ups that make use of different (or no) behavioural test batteries.

Primary outcome parameters and related animal procedures: Physical fitness tests (see appendix 1)
The physical fitness tests can be performed subsequent to the cognitive tests. We will investigate whether there are any correlations between physical fitness parameters and cognitive performance.

- Muscle strength will be measured using a grip strength meter. The mice are allowed to hold on to the meter, then the animal is pulled backwards until it releases the grid. Peak force is the primary outcome parameter to measure strength in the forelimbs or in all four limbs respectively.

- Stamina will be measured using the inverted screen test. Animals are placed on a grid, the grid is turned upside down and placed above a padded surface. The time until the animal has to let go of the grid and falls is taken as a measure for stamina.
- Endurance will be measured by running on a treadmill. At the end of lane on the treadmill there is a shock-grid which delivers a shock of ~0.3mA, which is used as a negative reinforce to induce running on the treadmill. In a progressively harder exercise test the animals run until exhaustion. Maximum running speed (Vmax) is taken as the primary outcome measure.
- Motor coordination will be tested in the balance beam test. During this test, the animal is placed on a thin beam or rod, which it has to cross to reach its home-cage. The time needed to cross the beam and the number of errors (paw-slips) will be taken as primary outcome parameters for coordination.

We use these outcome parameters because they allow us to test whether the (in)activity interventions have resulted in the expected changes in physical functioning in various domains, which will be affected differently by the different interventions.

Primary outcome parameters and related animal procedures: Cognitive tests
We will investigate the effects of the different interventions on different cognitive domains using learning and memory tasks that dependent on different learning and memory systems.

- WWWWtest. *(see appendix 2)* This task has been described in detail in literature[1] and is based on associative and sequential learning and recognition of novel objects and places. The primary outcome parameters for this test are successful discrimination of novel objects, novel locations of familiar objects and association of a certain context and familiar or novel objects.
- The automated Time-Place learning task. *(see appendix 2)* This task will be used to test time-place learning. This type of learning depends on a form of learning and memory which relies on the circadian system.[3] We will use a modified home-cage in which the food deprived animal is able to move around freely. In the upper part of the cage three platforms are located, which may or may not give access to a food reward depending on the time of day. The mouse will fall softly to the bottom of the cage when attempting to cross them at the wrong time of day. The percentage of correct choices is the main output parameter.

- The Morris Water Maze will be used to measure complex forms of spatial learning and memory. Mice are placed in a circular pool and learn to locate a hidden platform. The latency to find the platform as well as the path length to reach the platform will drop over the course of the acquisition trials. This is the primary outcome measure for acquisition of spatial memory. After 48 hours, the mice are placed back in the pool without a platform. The time spent by the mouse in the quadrant where the platform used to be located is the primary outcome measure for retention of spatial memory.
- Working memory will be tested in a Y-maze alternation task. The animal will be placed in a Y-maze, containing three identical arms separated at 120 degree angles from each other. When an animal has intact working memory it will remember which arm it has visited recently. It will explore the three arms more or less subsequently, visiting all three arms approximately the same number of times. The order of the arm exploration is an outcome parameter used to measure working memory.

Justification cognitive tests:
- Episodic memory has been shown to be affected by aging and AD in humans, and in mouse models of AD.[2,4] It is unknown whether exercise interventions can ameliorate these effects. The inclusion of both the object recognition based episodic memory tasks and the Time-Place learning task (dependent on the circadian system) is warranted because both test different forms of episodic memory, which rely on different memory systems. Giving the fast decline in circadian functioning that comes with age, it is important to include circadian system dependent episodic memory tasks.[5]
- These various learning and memory tests allows us to investigate how different memory systems are affected by exercise interventions, inactivity and cognitive stimulation. Performance in the simple object recognition tasks and the Morris Water Maze has been previously shown to be positively affected by voluntary running and an enriched environment and negatively affected by AD pathology in mice. It will therefore be of interest to see whether other forms of physical activity may affect the same learning and memory systems.

References:

[1] Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A. & Van Eldik, L. J. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front. Genet*. **5**, 1–23 (2014).

[2] Davis, K. E., Easton, A., Eacott, M. J. & Gigg, J. Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. *J. Alzheimers. Dis.* **33,** 681–98 (2013).

[3] [redacted]

[4] Dubois, B. *et al.* Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol.* **9,** 1118–1127 (2010).

[5] Kondratova, A. a & Kondratov, R. V. The circadian clock and pathology of the ageing brain. *Nat. Rev. Neurosci*. **13**, 325–35 (2012).

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

Disclaimer: The animal procedures and behavioural tests described here have been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use J20 AD-model mice.

(Exercise) Intervention methods

One of the inactivity methods and one of the strength exercise methods described in appendix 1 will be selected. Depending on whether the inactivity and strength methods are voluntary or forced, voluntary running or treadmill running will be used to serve as a model for endurance exercise.

Cognitive stimulation will be induced using an enriched environment. An enriched environment will consist of a larger cage filled with a number of objects and maze-like parts that are arranged differently every week. This environment will not contain a running wheel in the cognitive stimulation intervention, but will contain a running wheel in the combined intervention.[2]

All of these methods will be implemented for a period of 10-12 weeks before cognitive testing will commence.

Duration and frequency of physical fitness tests (As described in appendix 1)

- The grip strength test consists of two sessions: a total of four forelimb measurements and four forelimb & hind limb measurements. Each measurement takes approximately 10 seconds with breaks of 1 minute in between trials and a break of 2,5 minutes between sessions, adding up to a total duration of approximately 15 minutes.
- The inverted screen test starts with a 5 minute habituation period and consists of two trials with a 10 minute break in between trials. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests. Depending on how long the mouse can hold on to the screen, this test may take up to 50 minutes in total.
- Depending on the number of beams and rods that are included, the balance beam test can take 10-30 minutes. Addition of rods and beams that are of different width and shape can improve the ability of the test to discriminate between different exercise regimes. For every beam three trials will be performed, with 30 second breaks between them. Between two different beams, there will be a break of 1,5 minutes. This break has proven sufficient to prevent any possible effects of fatigue in previously performed tests.
- The treadmill endurance test consists of one trial. The duration of this trial is dependent on the endurance of the mouse and may take up to 45 minutes in total. The test is performed until exhaustion, which is classified as staying on the shock grid for 10 seconds despite prodding by the researcher. Early signs of fatigue are lowering of the tail and failing to run in the front part of the treadmill. Two days before the actual endurance test, a habituation trial of 12 minutes is performed to reduce novelty stress during the test.

Duration and frequency of cognitive tests (description of the tests is given in the part above)

- The different object recognition tasks will be tested over the course of 25 days, including 5 days of habituation.[1] These days include several habituation days, acquisition days and testing days. On each day, the animal will spend a total 10 minutes in the testing arena.

- The manual version of the time place learning test takes up to 21 days. We may assume the automated version will take as long, but this will need to be tested.
- In the Morris water maze an animal will be subjected to 4 trials (of maximum 8-10 minutes) per day, over the course of 5-7 days (until the animals has learned the task). Each trial will be finished when the mouse has located the platform and a trial. Probe tests will take place 48 hours after the last acquisition day, and will take 8 minutes per animal.
- The Y-maze alternation task takes a total of 8 minutes per animal.

Justification of selected approaches
The 10-12 weeks (exercise) intervention period is chosen based on literature and results from a first series of studies we performed.[1] Apart from the automated Time-Place learning task, the cognitive tests that are being performed have a standardized design which has been shown to give optimal results by our group and others. The protocol of the automated Time-Place learning task will be optimized using the results obtained from the experiments described in appendix 2. We will shorten the duration of this task as much as possible.

References:
[1] [redacted]
Unpublished results, [redacted]

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

Disclaimer: The animal procedures and behavioural tests described here have been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use J20 AD-model mice. Additional information is underlined for your convenience.

A mixed repeated measures design is possible for the physical fitness tests, but not for the cognitive tests (the performance of the same test twice poses a confounding factor because learning performing the same cognitive tasks in the same setting will become easier every time a cognitive test is repeated). A power analyses for a one-way ANOVA (based on a large effect size (f=0.4), an alpha of 0.05 and beta of 0.8) revealed a total of 80 mice is needed to test five groups (n=16 per group) and a total of 73 animals is needed to test four groups (n=19 per group, rounded up). This power analysis takes into account that we wish to investigate a possible interaction between genotype (J20 or wild-type) and (exercise) intervention type. The groups that are needed are listed in section B.

Because many of the interventions and the automated Time-Place learning test have not been used before, we cannot determine the exact expected effect sizes from literature (the above effect size is an educated guess based on voluntary running studies and studies performed with J20 mice). We will adjust our power analyses based on effect sizes and expected variation in primary outcome parameters based on studies that are being performed currently and the studies described in appendices 1 and 2.

Tissue analysis experimental set-up (3)
A power analyses for a one-way ANOVA (based on a larger effect size (f=0.5), an alpha of 0.05 and beta of 0.8) revealed a total of 53 mice is needed to test five groups (n=11 per group, rounded up) and a total of 48 animals is needed to test four groups (n=12 per group). The groups that are needed are listed in section B.

Pseudo-burrowers
We will need an additional group to control for certain elements of the strength exercise method depending on the strength exercise method that is suitable: the addition of pseudo-burrowers when the burrowing is our most promising method. We will adjust our power analyses based observations made (and data collected) in the studies that are being performed currently.[1]

[1] [redacted]

B. The animals

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

1. Male and female j20 AD-model mice[1,2] (C57Bl6J background) and C57Bl6J wild-type littermates (mus musculus), bred in our own facilities, 6 months old mice will be used. A maximum of n=602 J20 mice and 518 wild-type mice (most conservative estimates calculated below).

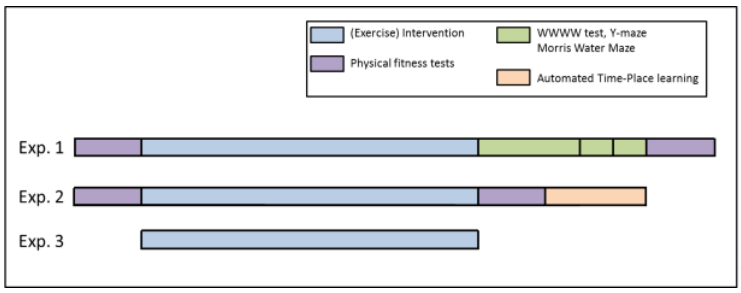
Disclaimer: Due to the similarity in set-up, calculations shown below are the similar compared to those shown in appendix 3. The two changes is that no aged mice are used in the currently described experiments and that we use J20 AD-model mice , additional information about these mice is underlined for your convenience.

Estimated numbers

Groups (A) - To investigate the effects of physical inactivity and various exercise regimes, groups of max. 18 wild-type or 21 J20 mice are needed:
1. Home cage controls
2a. Strength Exercise
2b. Pseudo strength exercise (if burrowing is strength exercise method)
3. Inactive
4. Endurance exercise (voluntary or treadmill running)
5. Endurance exercise + strength exercise

Groups (B) - To investigate differential effects of cognitive stimulation, aerobic exercise and a combination of both, groups of max. 21 wild-type or 25 j20 mice are needed:
1. Home-cage control
2. Enriched environment
3. Endurance exercise (voluntary running)
4. Endurance exercise + enriched environment (combined intervention)

Experimental set-up 1, 2 and 3 (see scheme on the right and section A) will be performed with groups A and B. So in total six experiments will be performed.



Because of practical limitations, we cannot test more than six of these groups in one experimental set-up. We need to perform three separate experiments: One to test for the effects of these interventions on Time-Place learning, one to test the effects in the other cognitive domains, one without behavioral tests for tissue collection. (see the first part of section A).

Calculations
- Groups of 16 or 19 wild-type mice are needed to test cognition of 5 or 4 groups under ideal conditions. If the selected exercise methods are voluntary, an additional 2 mice may need to be added to ensure at least 16 or 19 mice perform the desired behaviour (Based on our observations made for (resistance) running and burrowing).
- We expect to lose 15% of the J20 mice due to increased mortality which is part of the j20 phenotype.[3,4]

Therefore we will include max (16+2=) 18 or (19+2=) 21 animals per group when behavioral testing wild-type mice and (16+2+15%=) 21 or (19+2+15%=) 25 animals per group when behavioral testing in j20 mice.

- Finally, for both wild-type and j20 mice groups of mice that will not be tested in any of the cognitive tests will be included to collect brain tissue for immuno-histochemical analyses. (experimental set-up 3) These group sizes are smaller, but subject to the same alterations as the groups that are behaviourally tested: Addition of 2 animals for some voluntary methods and 15% mortality in J20 mice.

Therefore we will include max (11+2=) 13 or (12+2=)14 animals per group when testing wil-type mice or (11+2+15%=) 15 or (12+2+15%=)17 animals per group when testing in j20 mice.

In our most conservative scenario, which will be adjusted to include fewer animals wherever possible, this adds up to:

Groups (A) experimental set-up 1 + 2
- 5 groups*18 = 90 WT mice per experiment (=180)
Or, when Pseudo-Burrowing is added:
- 6 groups*18 = 108 WT mice per experiment (=216)
And:
- 5 groups*21 = 105 J20 mice per experiment (=210)
Or, when Pseudo-Burrowing is added:
- 6 groups*21 = 126 J20 mice per experiment (=256)

Groups (A) experimental set-up 3:
- 5 groups*13 = 65 WT mice
Or, when Pseudo-Burrowing is added:
- 6 groups*13 = 78 WT mice
And:
- 5 groups*15 = 65 J20 mice
Or, when Pseudo-Burrowing is added:
- 6 groups*15 = 78 J20 mice

Groups (B) experimental set-up 1 + 2
- 4 groups*21 = 84 WT mice per experiment (=168)
And
- 4 groups*25 = 100 J20 mice per experiment (=200)

Groups (B) experimental set-up 3:
- 4 groups*14 = 56 WT mice
- 4 groups*17 = 68 J20 mice

References
[1] Mucke, L. et al. High-level neuronal expression of abeta 1-42 in wild-type human amyloid protein precursor transgenic mice: synaptotoxicity without plaque formation. *J. Neurosci.* **20**, 4050–4058 (2000).
[2] Webster, S. J., Bachstetter, A. D., Nelson, P. T., Schmitt, F. A. & Van Eldik, L. J. Using mice to model Alzheimer's dementia: an overview of the clinical disease and the preclinical behavioral changes in 10 mouse models. *Front. Genet*. **5**, 1–23 (2014).
[3] https://www.jax.org/strain/006293 (Website of Jackson, the supplier of this mouse strain)
[4] Cheng, I. H., Scearce-Levie, K., Legleiter, J., Palop, J. J., Gerstein, H., Bien-Ly, N., … Mucke, L. (2007). Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. *J Biol Chem*, **282**(**33**), 23818–23828. http://doi.org/10.1074/jbc.M701078200

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'?

XNo

☐ 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.

Disclaimer: The information provided in section D has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use J20 AD-model mice. Additional information is underlined for your convenience.

Replacement: These experiments cannot be performed without the use of animals. The experiments require the use of complex animal models as they have to mimic the effects that the interventions may have in humans: There are no other models available that can be used to mimic the complex physiological adaptations in body (and brain) that are the consequence of cognitive and physical (in)activity. Moreover, no known *in silico* or *in vitro* models are available to mimic complex learning and

memory as is measured by the various cognitive tasks. Finally, the AD-like pathology displayed in the J20 mice can only partially be investigated in vitro. Plaque formation and the interaction between physiological changes induced by (exercise) interventions and this AD-like pathology can only be observed *in vivo* as this depends on complex physiological mechanisms with are only partially understood.

Reduction: We cannot reduce the number of animals any further due potential loss of power. We will minimize the number of animals needed as much as possible by optimizing the tests that are used as described in appendices 1 and 2. We will adjust our power analyses and therefore the group size according to the results of those studies.

Refinement: We aim to develop (exercise) interventions based on voluntary behaviour. This reduces stress due to handling as compared to forced methods. Habituation is used in behavioural tests. Furthermore, the shock intensity used in the treadmill test has been optimized in a previously performed pilot experiment and is set as low as possible (while still being a strong enough stimulus). The automated time-place learning paradigm is a refined version of the manually Time-Place learning paradigm: It does not require shocks as negative reinforcers, as was the case in the manual version.

Depending on the results of experiments described in Appendix 1, we may be able to characterize the effects of our interventions and exercise regimes mainly based on tissue analyses rather than behavioural testing (e.g. muscle weight instead of the grip strength test). We will need to confirm that this is possible in these studies, but if it is possible we will reduce the number of physical fitness tests as much as possible to further minimize discomfort (without compromising our read-out of physical fitness).

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

The behavioural tests will include habituation phases to reduce stress by handling animals or exposure to the test arena or the modified home-cage. Furthermore, all animals are habituated to handling and the behavioural test room before the start of behavioural testing.

We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

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.

Disclaimer: The information provided in section E has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use J20 AD-model mice. Additional information is underlined for your convenience.

A literature search was used to identify existing exercise methods and their effects on the AD-like phenotype. We found that the effects of strength exercise or induced inactivity have scarcely been tested in rodents and that there are no well-characterized (voluntary) strength exercise methods available for mice at this moment. The effects of exposure to an enriched environment and the effects of endurance exercise on spatial memory and AD-like pathological hallmarks have been tested before in AD mouse models. However, we need to include these groups in order to make a direct comparison between these existing interventions and our novel and combined interventions.

To date, no studies have investigated the effect of (endurance) exercise on episodic memory in Amyloid AD mice (like the j20 mice). The testing of Time-Place association has not been attempted in this AD-model either. Two studies were performed with the ‘’3xTgAD’’ mouse model (which shows tauopathy in addittion to Amyloid plaque formation). These studies showed episodic memory was affected by AD-like pathology, but did not investigate a possible effect of exercise interventions or inactivity.[1,2]

References:
[1] Davis, K. E., Easton, A., Eacott, M. J. & Gigg, J. Episodic-like memory for what-where-which occasion is selectively impaired in the 3xTgAD mouse model of Alzheimer's disease. J. Alzheimers. Dis. **33**, 681–98 (2013).
[2] Davis, K. E., Eacott, M. J., Easton, A. & Gigg, J. Episodic-like memory is sensitive to both Alzheimer's like pathological accumulation and normal ageing processes in mice. *Behav Brain Res* **254**, 73–82 (2013).

Accommodation and care

F. Accommodation and care

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

☐ No

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

The animals will be housed individually during the (exercise) intervention because it is necessary to monitor the physical activity of the individual. (e.g. amount burrowed or distance run)

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?

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

Animals may experience mild pain during the treadmill running test due to the mild electrical shock which is used as a negative reinforcer. This pain cannot be relieved, this would compromise the treadmill endurance test.

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

I. Other aspects compromising the welfare of the animals

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

Disclaimer: The information provided in section I has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use J20 AD-model mice.

Animals may be mildly stressed by the treadmill exercise and by our novel method of induced inactivity. They may be moderately stressed by the treadmill endurance test. During other behavioural tests animals may experience discomfort/novelty stress induced by handling and exposure to test arena's or apparatus. Moreover, animals that are used in the Time-Place learning paradigm will lose weight and

may experience mild stress or discomfort due to the food restriction or falling from the platforms on a soft surface.

Explain why these effects may emerge.

We expect no adverse effects on animal welfare during any of the voluntary exercise interventions, as these interventions are based on enriching the environment of the animal.

With regard to the inactivity methods, we may expect that the housing in a smaller cage or a cage without climbing possibilities will adversely affect the welfare of the animal to some extend as it cannot fully engage in natural forms of locomotor activity.

We expect treadmill running to induce mild stress induced by the shock grid at the end of the treadmill lanes. The treadmill endurance test may exert moderate discomfort because of running until exhaustion combined with the use of shocks.

Animals experience novelty stress when exposed to an unfamiliar environment, this is likely to occur in any of the test arena's we use. In the Morris Water Maze, some additional discomfort arises due to the forced swimming that is part of the maze protocol (mice are not particularly fond of swimming).

Finally, the food deprivation that is used to motivate the mice in the automated time-place learning task will induce adverse effects on the animals welfare due to a reduced caloric intake, weight loss and hunger.

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

The possible adverse welfare effects of the inactivity method cannot be avoided as this is an intrinsic part of the method. However, we will carefully monitor the inactivity groups to ensure they do not exert stereotypical behaviour (already when testing the method as indicated in appendix 1, but also during the experiments described in the current appendix). It is of note that our pilot experiments did not reveal that this temporary increase in inactivity causes abnormal overt behaviour.

The adverse welfare effects due to food restriction and falling during the automated Time-Place learning task cannot be avoided as this is an intrinsic part of the task and a food reward an essential positive reinforcer. We will monitor the weight and food intake of the animals that are being food deprived daily and provide enough food for their bodyweight not to fall below 85% of their original body weight.

A habituation phase is present in all our behavioural tests whenever it is possible, including the Morris Water Maze. This reduces stress due to handling and exposure to the tests.

A habituation phase can assure the animals get used to running in the treadmill at a low pace, reducing stress and discomfort to some extent. We cannot replace the foot shock as it is essential to motivate the animals. (Animals will habituate to and eventually ignore prodding by the researcher, for example.) Careful monitoring of the animals during the treadmill endurance test ensures they are placed back in their home cage as soon as they are exhausted.

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.

Disclaimer: The information provided in section J has been described in the exact same manner as in appendix 3 (copy-paste). This was done because this experiment is exactly the same in terms of set-up and behavioural tests being performed, the only difference is the fact that we use J20 AD-model mice. Additional information is underlined for your convenience.

An animal will be euthanized if a significant decline in general health status arises, indicated by for example a matt and lustreless fur, or by showing signs of pain. Mice will be euthanized if they lose more than 15% bodyweight, show piloerection or show abnormal behaviour, like a strong decline

feeding/drinking, and reluctance to accept handling. We do not expect the animals to reach such a condition as a consequence of the experiments, but adverse health effects may arise sporadically during the aging process of the mice.

Indicate the likely incidence.

None of these cases are to be expected in the wild-type mice. In the J20 mice we might expect ome morbidity related to the 15% increase in mortality, but the cause of premature death in this line is unknown (so we cannot be certain about increased morbidity).[1]

References:
[1] Cheng, I. H., Scearce-Levie, K., Legleiter, J., Palop, J. J., Gerstein, H., Bien-Ly, N., … Mucke, L. (2007). Accelerating amyloid-beta fibrillization reduces oligomer levels and functional deficits in Alzheimer disease mouse models. *J Biol Chem*, **282**(**33**), 23818–23828. http://doi.org/10.1074/jbc.M701078200

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').

Any of the behavioural tasks apart from the Time-Place learning test, because of novelty stress and the use of a mild shock in the treadmill test: 'Mild'. The automated time-place learning test, because of the food deprivation: 'Mild'

The treadmill endurance tests results in 'Moderate' discomfort due to the use of exhaustion as an endpoint in the test.

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.

All animals will be killed to collect muscle tissue, fat pads and the hearth, which are needed to confirm the physiological effects of the (in)activity intervention. Brain tissue will be collected to confirm the animals have developed AD pathology (Amyloid plaques).

The animals that undergo experimental set-up 3 (no behavioral testing) need to be killed in order to take out brain tissue for detailed (immuno-histochemical) analyses.

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

10 december 2014

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de bijbehorende toelichting, waarin elke stap in het beoordelingsproces wordt toegelicht

A. Algemene gegevens over de procedure

1. Aanvraagnummer (Intern RuG code **9005**)
2. Titel van het project: **Train the sedentary brain: Move smart to reduce the risk of dementia**
3. Titel van de NTS: **Train het inactieve brein: Slim bewegen om de kans op dementie te verminderen**
4. Type aanvraag:
   - ☐ **nieuwe aanvraag projectvergunning**
5. Contactgegevens DEC:
   - naam: DEC-RUG
   - telefoonnummer contactpersoon: [redacted]
   - mailadres contactpersoon: [redacted]
6. Adviestraject (data dd-mm-jjjj):
   - ☐ ontvangen door DEC: 08-10-2015
   - ☐ aanvraag compleet: 08-10-2015
   - ☐ in vergadering besproken: 15-10-2015
   - ☐ anderszins behandeld: 13-01-2016, 22-01-2016
   - ☐ termijnonderbreking(en) van / tot 19-10-2015 tot 17-12-2015, 13-01-2016 tot 19-01-2016
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen **n.v.t.**
   - ☐ aanpassing aanvraag: 19-12-2015, 19-01-2016
   - ☐ advies aan CCD: 26-01-2016
7. Eventueel horen van aanvrager **n.v.t.**
   - Datum
   - Plaats
   - Aantal aanwezige DEC-leden
   - Aanwezige (namens) aanvrager
   - Strekking van de vraag / vragen
   - Strekking van het (de) antwoord(en)

- Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag

8. Correspondentie met de aanvrager
   - Datum: 19-10-205, 13-01-2016
   - Strekking van de vraag / vragen:

Opmerkingen t.a.v. de projectbeschrijving

Op pagina 3, bij 3.2. Doel, schrijft u 'five research questions will be answered' . Hierna volgt een lijst met 4 (en niet 5) onderzoeksvragen.

De achtergrondbeschrijving (# 3.1) in het projectvoorstel is een vrij slordig en te lang stuk tekst met veel intrinsieke herhalingen. Het primaire doel van het hele project wordt al vrij snel duidelijk, maar dan volgen een reeks uitspraken/veronderstellingen zonder enige referentie uit de literatuur, inclusief eigen werk. Dit stuk tekst zal het eerste zijn dat door het CCD-bureau gelezen wordt – en dat moet een heldere binnenkomer zijn. Lange zinnen en/of vrij cryptische zinnen kunnen beter vermeden worden.

Voorbeelden hiervan zijn:

' Inactivity is a risk-factor which is independent of exercise engagement with regard to the development of cardiovascular diseases and possibly neurodegenerative diseases.'

'Translational value
Of course it is of importance to ensure results from animal studies are of translational value (to the clinical setting). In order to ensure we can link any causal mechanisms identified in mice to the human situation, this research is performed in a consortium consisting of our research group and three research group performing similar experiments in elderly and dementia patients (Including cohorts of APOE4 carriers in both groups).'

In aanvulling op hierboven, de vervolginformatie dat het project deel uitmaakt van een consortium is uiterst relevant om de geloofwaardigheid van het projectvoorstel te onderbouwen, maar nadere informatie wordt niet gegeven. Bij 'Vragen t.a.v. de projectbeschrijving' (zie hieronder) wordt u hierover ook een vraag gesteld.
Een ander voorbeeld is: Bij 'Achievability' schrijft u: 'We do not anticipate any technical difficulties as our research group is quite experienced in performing behavioural studies and exercise interventions in rodents. We've recently developed novel strength exercise models and a novel inactivity model in mice. Combined with existing (exercise) models in mice, such as voluntary running in a running wheel or housing in an enriched environment, these models will allow us to test for independent and synergistic effects of inactivity, endurance exercise, strength exercise and cognitive stimulation in mice'

Als deze zin met de kritische/sceptische blik van een beoordelende instantie zou worden bekeken dan staat hier: - dat we geen technische problemen verwachten - want we zijn heel ervaren - en we hebben net krachttraining-modellen en een inactiviteitsmodel opgezet, en samen met wat we al hebben, zoals loopwielen en kooiverrijking, maken deze modellen het mogelijk 'to test for independent and synergistic effects of inactivity, endurance exercise, strength exercise and cognitive stimulation in mice.'
Er is overtuigender argumentatie voorstelbaar. Overigens, synergisme betekent: 'meer dan de som der delen'; mogelijk zou een additief effect al heel mooi zou zijn.
Voor bovenstaand deel kan gesteld worden dat de marketing van de eigen expertise en

wetenschappelijke positie (referenties!) een flink stuk beter kan. Ook onder # 3.3 (Belang) worden een aantal uitspraken gedaan die smeken om referenties – en tevens om een compactere tekst.
Rubriek # 3.4 (Onderzoeksstrategie) begint met de zin:
'Before we perform the experiments designed to meet the objectives specified in part 3.2 we need to meet the following prerequisites:
1. A working model for strength exercise in aged mice and AD model mice.
2. A working model of inactivity in aged mice and AD model mice.
3. A working cognitive test battery, including an episodic memory test to test higher cognitive functions, which can be performed successfully by aged mice.'
en dan volgt: 'We are currently comparing a number of strength and inactivity models in order to select the most effective models, these studies are performed in healthy young male mice. Similarly, our automated Time-Place learning test has been applied in healthy young male mice. This test needs further optimization though'.

Bij bovenstaande is het duidelijk wat er aan modellen nodig is maar wat er al loopt blijft onduidelijk. In de lange tekst die volgt komt herhaaldelijk naar voren dat iets nog opgezet of ontwikkeld of geoptimaliseerd wordt of moet worden.

Vragen t.a.v. de projectbeschrijving

Vraag 1: U noemt dat het werk deel uitmaakt van een consortium waarin ook klinische studies bij ouderen en demente patiënten plaatsvinden. Kunt u de relatie/translatie van het dierexperimentele werk met het (mogelijk) onderzoek bij de mens beter beschrijven?

Vraag 2: De mogelijkheid bestaat dat overmatige activiteit (b.v. door aanbieden van loopwiel) leidt tot verslaving, hetgeen een invloed kan hebben op de te bestuderen parameters. Hoe ziet u dit?

Opmerkingen t.a.v. de bijlagen
Appendix 1 (Optimization of mouse models of strength exercise and physical inactivity) (suggestie: laat optimization eruit) bevat verschillende paragrafen die duidelijk en concreet zijn maar te vaak wordt aangegeven dat er nog van alles op bruikbaarheid uitgetest of geoptimaliseerd moet worden. Een berekening of validatie van aantallen dieren is nagenoeg afwezig.
Appendix 2 (Optimization of episodic memory tests in mice). Dit moet korter, minder voorwaardelijk (if these tests are successful (2x), minder verbrokkeld (de WWWW-test wordt 2x uitgelegd, de eerste keer met het accent op potentiële problemen) – Positief is hier het gebruik van referenties (globaal aangegeven). Aantallen dieren zijn wederom niet gevalideerd.
Appendix 3 (Effects of endurance exercise, strength exercise, combination therapy and physical inactivity in young and aged mice) (suggestie: laat combination therapy eruit, vraagt om misverstanden). Opmerkelijk is hier: copy-paste van drie tekstblokken uit appendices 1 en 2, en dat kan natuurlijk niet. De technische beschrijving van nog niet eerder ge-expliciteerde modellen lezen vrij goed weg, maar allerlei uitspraken vragen om referenties.
Dieraantallen worden genoemd maar niet gevalideerd.
Appendix 4 (Effects of endurance exercise, strength exercise, combination therapy and physical inactivity in J20 Alzheimer mice) is nagenoeg geheel copy-paste van appendix 3. Overigens wordt hier voor het eerst de J20 Alzheimer muis genoemd, helaas zonder enige (korte) beschrijving van de eigenschappen (literatuur!). Dieraantallen genoemd, verder niet.
Appendix 5 (Effects of endurance exercise, strength exercise, combination therapy and physical inactivity in hAPOE4 mice). Voor 99% copy-paste van appendix 4. Dieraantallen niet gevalideerd.

Vervolgvragen

De algemene project-tekst is verbeterd, is helderder en is inclusief referenties. Wat nog steeds mist is de link met het parallelle humane onderzoek. Dit staat wel in de respons op de vragen maar niet of nauwelijks (expliciet) benoemd in de tekst.

Het bevat een go/no go strategie (test ontwikkelen en dan testen in doelgroep dieren). Het is echter niet duidelijk hoe groot u de kans inschat dat de eerste stap lukt en dat de tweede dan zo groot kan worden ingezet. Verder staan in 1 en 2 al oude en AD dieren genoemd; dit is niet helder in de rest van de tekst. De tekst suggereert dat dit alleen in 3,4,5 gebeurt.

De aantallen dieren staan nog wat ruim de NTS: 3000-4000 is nogal een marge.
De opgegeven aantallen in de appendices lijken wel goed onderbouwd door power berekeningen. De marge in de totale aantallen in de NTS zou mogelijk kunnen voortvloeien uit de ruimte die u als aanvrager neemt om een extra controle groep in te zetten in het merendeel van de experimenten. Het Zou ook kunnen komen doordat in de eerste appendices de mogelijkheid wordt genoemd dat het experiment in tweevoud gedaan moet worden. Mogelijk kan de tekst in de NTS nog aangepast/verduidelijkt worden op het punt van de totale aantallen.

- Datum antwoord: 17-12-2015, 19-01-2016
- Strekking van het (de) antwoord(en): **De gevraagde verduidelijkingen zijn verwerkt in het projectvoorstel en de bijlages. 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 (dierproeven in de zin der wet)
2. De aanvraag betreft een nieuwe aanvraag
3. De DEC is competent om hierover te adviseren
4. Vanwege betrokkenheid bij het betreffende project is een aantal DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, niet betrokken bij de advisering **NVT**

C. Beoordeling (inhoud):

1. Het project is:

   - **X uit wetenschappelijk oogpunt verantwoord**

- uit onderwijskundig oogpunt verantwoord
- uit het oogpunt van productiedoeleinden verantwoord
- wettelijk vereist

2. De in de aanvraag aangekruiste doelcategorie(ën) is / zijn in overeenstemming met de hoofddoelstelling(en) **JA, deze zijn juist aangekruist.**
3. De DEC onderschrijft het belang van de doelstelling. Het wordt ingeschat als **substantieel**
4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project **JA, met de gevolgde strategie en aanpak zullen de doelstellingen van het project behaald kunnen worden.**
5. Er is sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren. De keuze hiervoor is voldoende wetenschappelijk onderbouwd **NVT**
6. Het ongerief als gevolg van de dierproeven is realistisch ingeschat en geclassificeerd **JA, de inschatting is realistisch en goed geclassificeerd.**
7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen, want inspanning wordt gebruikt als manier om veroudering/dementia tegen te gaan/te beinvloeden. Dit is gekoppeld aan het aanpalende humane onderzoek. Dit kan niet anders dan in levende dieren. Verder wordt gedrag (cognitieve vaardigheden) gebruikt als uitleesparameter voor de behandelingen. Dit kan wederom niet anders dan in levende dieren.**
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. De aanvrager beschikt over voldoende expertise en informatie om, bij wettelijk vereist onderzoek, te voorkomen dat onnodige duplicatie plaatsvindt **JA, de onderzoekers hebben een go/no go moment ingebouwd om te voorkomen dat er onnodig veel dieren worden gebruikt in het experiment. Verder is de statistische onderbouwing van de aantallen adequaat. De onderzoeksgroep bevat de nodige expertise. Een sterk punt van het project is de koppeling met parallel lopend onderzoek bij mensen.**
9. Het project is in overeenstemming met de vereiste van de **verfijning** van

dierproeven en het project is zo opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Er is geen sprake van belangwekkende milieueffecten **JA, de onderzoekers hebben het onderzoek zodanig opgezet dat het project in overeenstemming is met de vereisten van verfijning. Bijvoorbeeld in Bijlage 1 geven de onderzoekers aan dat waar dat mogelijk is voor de inspanningstesten geen geforceerde methoden worden gebruikt ('voluntary'), dat dieren worden gehabitueerd aan opstellingen om (onnodige) stress te voorkomen, en dat de shock intensiteit bij de 'treadmill test' zo laag mogelijk is voor het beoogde doel.**

10. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd **JA, het is voor iedereen een goed leesbare tekst.**

D. Ethische afweging

Dementie, in het bijzonder de ziekte van Alzheimer, is een ernstige neurocognitieve (verouderings)aandoening, die de kwaliteit van het leven (zeer) negatief beinvloedt. Onderzoek bij mens en dier, dat de kans kan verlagen of de impact kan verminderen, is daarom van groot maatschappelijk/gezondheidsbelang. Dit dierexperimentele onderzoek is gericht op de rol van verschillende vormen van beweging(sport) in het voorkomen van/beïnvloeden van dementie, zoals de ziekte van Alzheimer. Het onderzoek is gekoppeld aan en ingebed in een groter humaan-georienteerd onderzoeksproject gericht op dementie. Het heeft daarom een grote kans succesvol te zijn omdat directe afstemming en uitwisseling met aanpalend humaan onderzoek mogelijk is. Verder hebben de onderzoekers een go/no go moment ingebouwd om te voorkomen dat zonder degelijke onderbouwing verdere experimenten worden gedaan. Het matige ongerief dat de dieren wordt aangedaan wordt in de visie van de DEC moreel gerechtvaardigd vanwege het maatschappelijk/gezondheidsbelang en de verwachte resultaten. Deze rechtvaardiging vindt verder zijn grond in de zorgvuldige methodische en stapsgewijze benadering van het project.

E. Advies

1. Advies aan de CCD

   - ☐ **X De DEC adviseert de vergunning te verlenen**

2. Het uitgebrachte advies is gebaseerd op **consensus**

10

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Rijksuniversiteit Groningen
[redacted]
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9713 AV GRONINGEN

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
AVD105002016405

Bijlagen
2

Datum 26 januari 2016
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte [redacted],
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 26 januari 2016.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD105002016405. 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:	10500
Naam instelling of organisatie:	Rijksuniversiteit Groningen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	1179037
Straat en huisnummer:	A. Deusinglaan 1 [redacted]
Postcode en plaats:	9713 AV GRONINGEN

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

Gegevens plaatsvervangende verantwoordelijke onderzoeker

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

Over uw aanvraag

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

Over uw project

Geplande startdatum:	1 december 2015
Geplande einddatum:	1 december 2020
Titel project:	Train the sedentary brain: Move smart to reduce the risk of dementia
Titel niet-technische samenvatting:	Train het inactieve brein: Slim bewegen om de kans op dementie te verminderen
Naam DEC:	DEC-RUG
Postadres DEC:	A. Deusinglaan 1, [redacted]
E-mailadres DEC:	secrdec.umcg@umcg.nl

Betaalgegevens

De leges bedragen:	€ 1727,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

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

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Groningen

Centrale Commissie Dierproeven

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Aanvraagnummer
AVD105002016405

Bijlagen
2

Datum 26 januari 2016
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 26 januari 2016
Vervaldatum: 25 februari 2016
Factuurnummer: 16700405

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD105002016405	€ 1727,-

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

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD105002016405
Bijlagen
1

Datum 25 FEB. 2016
Betreft Beslissing Aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 26 januari 2016 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Train the sedentary brain: Move smart to reduce the risk of dementia" met aanvraagnummer AVD105002016405. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de algemene voorwaarden zoals genoemd in de vergunning.
U kunt met uw project "Train the sedentary brain: Move smart to reduce the risk of dementia" starten. De vergunning wordt afgegeven van 25 februari 2016 tot en met 1 december 2020. De looptijd van de vergunning wijkt af omdat de startdatum in de aanvraag in het verleden ligt.
Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-RUG gevoegd. Dit advies is opgesteld op 26 januari 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. Wij nemen het advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering. Met het oog op art. 10a, lid 1, worden twee algemene voorwaarden toegevoegd. Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

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

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

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

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

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

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peuter
Algemeen Secretaris

Bijlagen:
- Niet technische samenvatting
- 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:	Rijksuniversiteit Groningen
Adres:	A. Deusinglaan 1 HPC: FA29
Postcode en plaats:	9713 AV GRONINGEN
Deelnemersnummer:	10500

deze projectvergunning voor het tijdvak 25 februari 2016 tot en met 1 december 2020, voor het project "Train the sedentary brain: Move smart to reduce the risk of dementia" met aanvraagnummer AVD105002016405, volgens advies van Dierexperimentencommissie DEC-RUG.
De functie van de verantwoordelijk onderzoeker is [redacted]
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 26 januari 2016
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 26 januari 2016;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 26 januari 2016;
c Advies van dierexperimentencommissie d.d. 26 januari 2016, ontvangen op 26 januari 2016.

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
Testing of strength exercise methods and physical inactivity methods in mice.	Muizen (Mus musculus) / C57BL/6; J20 (Alzheimer's Ziekte model); volwassen en oudere dieren; beide geslachten	392	Matig / moderate	
Testing of episodic memory tasks in mice	Muizen (Mus musculus) / Gelijk aan dierproef 1.	146	Licht / mild	De dieren worden individueel gehuisvest.
Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in adult and aged mice.	Muizen (Mus musculus) / C57BL/6; volwassen en oudere dieren; beide geslachten	1096	Matig / moderate	De dieren worden individueel gehuisvest.
Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in hAPOE4 mice.	Muizen (Mus musculus) / hAPOEe4 en hAPOEe3; oudere dieren; vrouwelijk	1156	Matig / moderate	De dieren worden individueel gehuisvest.
Effects of endurance exercise, strength exercise, physical inactivity and cognitive stimulation in Amyloid Alzheimer's Disease model mice.	Muizen (Mus musculus) / J20; C57BL/6; volwassen dieren; beide geslachten	1120	Matig / moderate	De dieren worden individueel gehuisvest.

Voorwaarden
Op grond van artikel 10a1 lid 2 Wod zijn aan een projectvergunning voorwaarden te stellen
De vergunning wordt verleend onder de voorwaarde dat de go/no go momenten worden afgestemd met de Instantie voor Dierenwelzijn.

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

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.

[redacted]

Van: Info-zbo
Verzonden: donderdag 25 februari 2016 14:01
Aan: [redacted]
Onderwerp: terugkoppeling besluit aanvraag AVD105002016405

Geachte leden van DEC-RUG,

Op 26 januari 2016 heeft u advies uitgebracht op de projectaanvraag met titel 'Train the sedentary brain: Move smart to reduce the risk of dementia', en aanvraagnummer AVD105002016405. Wij danken u voor uw advies en koppelen graag het oordeel van de CCD over deze aanvraag aan u terug. De CCD heeft besloten de vergunning te verlenen, onder de volgende algemene voorwaarden:

De vergunning wordt verleend onder de voorwaarde dat de go/no go momenten worden afgestemd met de Instantie voor Dierenwelzijn.

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

De aanvrager en de verantwoordelijk onderzoeker zijn hierover ingelicht.

We hopen u op deze wijze voldoende geïnformeerd te hebben.

Met vriendelijke groet,

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