	Inventaris Wob-verzoek W16-04s								
			wordt verstrekt			**weigeringsgronden**			
nr.	**document**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
	NTS 20151210								
1	Aanvraagformulier						x	x	
2	Brief mbt factuurinformatie						x	x	
3	Ontvangstbevestiging						x	x	
4	Beschikking en vergunning						x	x	
5	Niet-technische samenvatting	x							
6	Projectvoorstel						x	x	
7	Bijlagen dierproeven			x					
8	Appendix 3 en 4			x				x	
9	Tabel breeding			x					
10	Appendix 1 en 2			x					
11	Tabel year			x					
12	DEC advies				x		x	x	
13	Advies CCD		x						x

1.

1 1 AUG 2015

Aanvraag

Projectvergunning Dierproeven

Administratieve gegevens

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

1 Gegevens aanvrager

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

☑ Ja > *Vul uw deelnemernummer in* 10300

☐ Nee > *U kunt geen aanvraag doen*

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

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

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

Straat en huisnummer	Geert Grooteplein-Noord 9
Postbus	9102
Postcode en plaats	6525EZ Nijmegen
IBAN	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer	UMC St Radboud

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

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

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

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
		Functie	Instantievoor Dierenwelzijn
		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	07-09-2015
		Einddatum	07-09-2020
3.2	Wat is de titel van het project?	Language related genes in neurodevelopment and brain function	
3.3	Wat is de titel van de niet-technische samenvatting?	Onderzoek naar de invloed van taalgenen op de hersenontwikkeling en hersenfunctie	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	RU DEC
		Postadres	Postbus 9101, 6500 HB Nijmegen (627 DEC B4)
		E-mailadres	

4 Betaalgegevens

4.1	Om welk type aanvraag gaat het?	☑ Nieuwe aanvraag Projectvergunning € 741,00 Lege ☐ Wijziging € Lege
4.2	Op welke wijze wilt u dit bedrag aan de CCD voldoen. *Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.*	☐ Via een eenmalige incasso ☑ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

- ☒ Projectvoorstel
- ☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

- ☐ Melding Machtiging
- ☒ DEC advies, document factuurgegevens

6 Ondertekening

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

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag

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

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

Naam	
Functie	Instantie voor dierenwelzijn
Plaats	Nijmegen
Datum	07-08-2015
Handtekening	

10300

Radboud universitair medisch centrum
Concernstaf, sectie Kwaliteit en Veiligheid

Postbus 9101, 6500 HB Nijmegen
Huispost 628
Geert Grooteplein 10

www.radboudumc.nl

KvK 41055629/4

Geert Grooteplein 10
Postbus 9101
6500 HB Nijmegen

Datum
7 augustus 2015

Instantie voor Dierenwelzijn

Onderwerp
Factuurinformatie Projectaanvraag

Geachte CCD,

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

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

Factuuradres:	Radboudumc 28 F&A crediteuren Postbus 9101 6500HB, Nijmegen
Kostenplaats en kostensoort:	040823-461220
CDL projectnummer:	2015-0038
Verantwoordelijk onderzoeker:	

Bij voorbaat dank.

Met vriendelijke groeten

Instantie voor Dierenwelzijn

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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

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

Onze referentie
Aanvraagnummer
AVD103002015210

Bijlagen
2

Datum 13-08-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Geachte heer/mevrouw [redacted]
Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 11 augustus 2015.
Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD103002015210. Gebruik dit nummer wanneer u contact met de CCD opneemt.

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

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

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

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA:	10300
Naam instelling of organisatie:	Stichting Katholieke Universiteit Nijmegen
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	41055629
Straat en huisnummer:	Geert Grooteplein 9
Postbus:	9102
Postcode en plaats:	6525 EZ NIJMEGEN
IBAN:	NL90ABNA0231209983
Tenaamstelling van het rekeningnummer:	UMC St Radboud

Gegevens verantwoordelijke onderzoeker

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

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:

Functie:

Afdeling:

Telefoonnummer: 0

E-mailadres:

Gegevens verantwoordelijke uitvoering proces

Naam:

Functie: Instantie voor Dierenwelzijn

Afdeling:

Telefoonnummer:

E-mailadres: instantievoordierenwelzijn@radboudumc.nl

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: 7 september 2015

Geplande einddatum: 7 september 2020

Titel project: Language related genes in neurodevelopment and brain function

Titel niet-technische samenvatting: Onderzoek naar de invloed van taalgenen op de hersenontwikkeling en hersenfunctie

Naam DEC: RU DEC

Postadres DEC: Postbus 9101, 6500 HB Nijmegen (

E-mailadres DEC:

Betaalgegevens

De leges bedragen: € 741,-

De leges voldoet u: na ontvangst van de factuur

Checklist bijlagen

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

Ondertekening

Naam:	
Functie:	Instantie voor dierenwelzijn
Plaats:	Nijmegen
Datum:	7 augustus 2015

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

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

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

Onze referentie
Aanvraagnummer
AVD103002015210

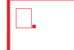
Bijlagen
2

Datum 13-08-2015
Betreft Ontvangstbevestiging Aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 13 augustus 2015
Vervaldatum: 12 september 2015
Factuurnummer: 201570210

Omschrijving	**Bedrag**
Betaling leges projectvegrunning dierproeven Betreft aanvraag AVD103002015210	€ 741,00

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



Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Stichting Katholieke Universiteit Nijmegen
)
Postbus 9102
6525EZ Nijmegen

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-2800028 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD103002015210

Bijlagen
1

Datum 21 september 2015
Betreft Beslissing aanvraag projectvergunning dierproeven

Geachte mevrouw

Op 8 augustus 2015 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'Language related genes in neurodevelopment and brain function' met aanvraagnummer AVD103002015210. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning. De onderzoeker zal zowel de go/no-go momenten als de criteria om een optionele behandeling uit te voeren met de IvD afstemmen. U kunt met uw project 'Language related genes in neurodevelopment and brain function' starten. De vergunning wordt afgegeven van 21 september 2015 tot en met 7 september 2020.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie RUDEC gevoegd. Dit advies is opgesteld op 6 augustus 2015. 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 dit advies van de commissie over, inclusief de daaraan ten grondslag liggende motivering.
Dit advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving liggen ten grondslag aan dit besluit.

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

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze 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.zbo-ccd.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Met vriendelijke groet,

Centrale Commissie Dierproeven
namens deze:

ir. G. de Peuter
Algemeen Secretaris

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

Bijlagen
- Vergunning
    - Hiervan deeluitmakend:
        - DEC-advies
        - Weergave wet en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Stichting Katholieke Universiteit Nijmegen
Adres:	Geert Grooteplein-Noord 9
Postcode en woonplaats:	6525EZ Nijmegen
Deelnemersnummer:	10300

deze projectvergunning voor het tijdvak 21 september 2015 tot en met 7 september 2020, voor het project 'Language related genes in neurodevelopment and brain function' met aanvraagnummer AVD103002015210, volgens advies van Dierexperimentencommissie RUDEC.
De functie van de verantwoordelijk onderzoeker is [redacted]
De aanvraag omvat de volgende bescheiden:

1. een aanvraagformulier projectvergunning dierproeven, ontvangen per post op 11 augustus 2015;
2. de bij het aanvraagformulier behorende bijlagen:
   a. Projectvoorstel, zoals ontvangen bij digitale indiening op 8 augustus 2015;
   b. Niet-Technische Samenvatting van het project, zoals ontvangen bij digitale indiening op 8 augustus 2015;
   c. Advies van dierexperimentencommissie RUDEC d.d. 6 augustus 2015 en ontvangen op 8 augustus 2015.

Dierproeven

Naam dierproef	Diersoort	Aantal dieren voor het vergunde tijdvak	Ernst
Stock breeding without discomfort and organ extraction	Muizen (*Mus musculus*); C57BL6J;	5680	Licht en terminaal
Breeding with discomfort and organ extraction	Muizen (*Mus musculus*); C57BL6J;	3400	Licht, matig en terminaal
Substance administration and organ extraction	Muizen (*Mus musculus*); C57BL6J;	2760	Matig en terminaal.
Behavioral testing and organ extraction	Muizen (*Mus musculus*); C57BL6J;	1440	Licht

Voorwaarde:
Op grond van artikel 10a1 lid 2 Wet zijn aan een projectvergunning voorwaarden te stellen:

De onderzoeker zal zowel de go/no-go momenten als de criteria om een optionele behandeling uit te voeren met de IvD afstemmen.

In Artikel 10, eerste lid, onder a, Wet op de dierproeven, 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 van Economische Zaken een ontheffing is verleend.

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

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

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

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

Form
Project proposal

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen
1.3	Provide the title of the project.	Language related genes in neurodevelopment and brain function

2 Categories

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

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

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

3 General description of the project

3.1 Background

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

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

Neurodevelopmental syndromes in which the proper establishment of functional language is disrupted— such as autism, developmental dyspraxia and speech/language impairments - have a major impact on the educational, social and mental wellbeing of modern society. Extensive evidence indicates the importance of multifactorial genetic causes underlying these disorders. Knowledge of both the neuronal and molecular mechanisms that are disturbed in language-related disorders can lead to improvements for affected people, including early diagnosis of those at risk, and development of novel therapies. In the last decade, researchers have begun to investigate quantitative associations between particular candidate gene loci and common cases of disrupted language skills, and there have been notable successes in uncovering the molecular background of multiple disorders with a language-related phenotype (For review, see Genetics of speech and language disorders, Annu. Rev. Genomics Hum. Genet. 2011. 12:145–64 and Neurogenomics of speech and language disorders: the road ahead. Deriziotis and Fisher Genome Biology 2013, 14:204)
A particularly striking example is the identification of mutations in the gene FOXP2, which lead to a severe speech and language disorder - developmental verbal dyspraxia - in carriers of one affected allele.
In the past 15 years, extensive research has been conducted by multiple research groups on the molecular effects of FOXP2 mutations. FOXP2 is known as a transcription factor, a class of proteins which regulate transcription of RNA from DNA. Evidence for interaction of FOXP2 with multiple genes important for normal brain function has been established. For a comprehensive review of the role of FOXP2 in the brain see e.g. FOXP2 as a molecular window into speech and language. Trends in Genetics 2009 4:166-177 and: What can mice tell us about Foxp2 function? Current Opinion in Neurobiology 2014, 28:72–79. In summary, the transcription factor FOXP2 is implicated in multiple processes relevant for proper brain function, such as neuron outgrowth and establishment of proper connectivity. The FOXP2 as a molecular window into speech and language review focuses on FOXP2 in humans, its history, interactions with other proteins and evidence from molecular and behavioural science on FOXP2. The what can mice tell us about Foxp2 function?, review focuses on Foxp2 in mouse and what mouse models for FOXP2 can teach us about language. This more recent paper highlights the recent molecular, cellular and behavioural evidence for Foxp2 on a single mouse model, but expands on this knowledge and explains the construct validity of the mouse as a model for Foxp2.

Additionally, it has been shown that FOXP2 is very well conserved throughout the animal kingdom with homologs of the human FOXP2 gene found in fruitflies, fish, birds, rodents, bats and large primates, lending construct validity to our mouse studies. This conservation is very well described in: The eloquent ape: genes, brains and the evolution of language, Nature Reviews Genetics 2006, 1:9-20. The co-applicant's group ([redacted] [redacted] has enabled production and maintenance of several genetically modified mouse lines with mutations mirroring those found in monogenic speech disorders in humans. The goals and aims in this project proposal are supported by multiple grants and funding agencies on both a national and international level. These include departmental funding from the [redacted] Gesellschaft, funding from the [redacted] and multiple Marie Curie career integration grants.
The mouse lines have previously been used to investigate gene-networks affected by Foxp2 mutations as well as the spatiotemporal expression pattern of Foxp2 in the brain. These results have opened up a very interesting entry point to study how Foxp2 affects the brain and will also enable us to advance our investigations into different monogenic disorders in which speech and language are affected. Thus, this project aims to combine the genetic expertise of [redacted] with the interests of the [redacted] of the [redacted] Utilizing our combined experience, we will be able to design the best possible experiments in order to answer how mutations in language-related genes affect the brain on different levels.
Previous research (Foxp2 Regulates Gene Networks Implicated in Neurite Outgrowth in the Developing Brain, PLoS genetics 2011, 7:e1002145) has shown intriguing evidence on both the genetic and functional level implicating Foxp2 in neuronal outgrowth and development. Very recently, another role for Foxp2 in the regulation of retinoic acid receptors has been found by the same group, implicating Foxp2 in regulation of cellular activity as well (FOXP2 drives neuronal differentiation by interacting with retinoic acid signaling pathways , Frontiers in cellular neuroscience 2014, 8:305:1-13). We want to further investigate these roles of Foxp2 and also extrapolate to other monogenic disorders with a speech and language phenotype. From these previous results, we have generated the hypothesis that Foxp2 mutation and other disorders affecting speech and language affect specific cell types and brain regions. Specifically, cells present in brain regions involved in motor coordination such as cortex, striatum, thalamus and cerebellum (Characterization of Foxp2 and Foxp1 mRNA and protein in the developing and mature brain, The journal of comparative neurology 2003, 2:266-279). Preliminary results of our lab have shown that a specific cell population (Dopamine-1 receptor expressing cells) in the mouse striatum is specifically affected when functional Foxp2 is decreased or removed. Our goal is to find the mechanistic links between the gene defect and behavioural phenotype. To this end, we will need to investigate different aspects of motor network function: The molecular, cellular, physiological and behavioural levels.
Proper connectivity within and between brain structures during development is essential for correct brain function, especially for complex behaviors such as speech production and language. A recent review (Corticostriatal connectivity and its role in disease, Nature Reviews Neuroscience 2013, 4:278-291) very elegantly explains that defects in specific cell populations along the brain's motor circuits are a common cause of motor disorders. In addition, the importance of proper brain wiring for movement is described, which is in turn essential for the acquisition of motor control over facial muscles necessary to produce functional language.
By using mouse models for genes implicated in the development of speech, we can elucidate the effects these genes have onto the brain in exquisite detail. Specifically, mouse models allow close investigation of the development and function of the brain. Previous molecular research conducted in the MPI language and genetics department has shown that mutations found in FOXP2 affect the proper development of cultured mouse neurons and neuron-like cell lines. As explained before, we have ample experience with multiple mouse lines implicated in speech and language disorders.
Research utilizing these models enables us to understand how gene mutations affect the individual. Effects can range from molecular mechanisms to

behavioral effects, and we propose to study those in the experimental paradigms outlined here. We utilize different approaches and experimental techniques to investigate how the brain's capacity to execute complex tasks such as complex motor sequences is established, and how disorder-causing mutations affect the brain.
Another aspect which has not yet been investigated is the potential for rescue of the phenotype shown in our mouse models. We have developed the hypothesis that the motor learning deficits we observe in our mouse models are caused, for the most part, by defects in specific cell populations in the brain's motor circuits. Thus, we plan to ultimately use advanced methods such as targeted optogenetics to adjust the activity of those cell populations, thereby remedying the motor learning phenotype. This will further our understanding of the necessity of Foxp2 and how possible treatment options can be established for people suffering from similar speech and language problems.
In addition to these new directions in Foxp2 specific research, we would like to extend our research aims to other genes implicated in speech and language disorders. Research towards discovering new genes is still ongoing, but first candidate genes for our research are genes such as Foxp1, Tbr1 and Cntnap2, which are all implicated in different cognitive deficits with a prominent language phenotype.
FoxP1 is another member of the forkhead box group of proteins to which FoxP2 also belongs. These proteins all share the same characteristics, being DNA binding transcription factors. Interestingly, FoxP1 knockout mice are embryonal lethal, indicating FoxP1 as a very impart gene during development. Mice with a (tissue specific) conditional knockout of FoxP1 later in development show behavioral problems similar to FoxP2 in several aspects, as well as morphological changes in brain regions where FoxP2 is highly expressed. The possible functional significance of FoxP1 and the relevance of investigating FoxP1 malfunction in the light of FoxP2 research is well described in: Brain-specific Foxp1 deletion impairs neuronal development, 2015, Molecular Psychiatry 49:632-639.
Tbr1 is another transcription factor expressed in the brain and known to be important during brain development. Mutations in Tbr1 lead to autism like social behavior in humans and mice. Additonally, in mice a negative impact on ultrasonic vocalizations has been reported. Moreover, it has been shown to interact strongly with FoxP2 in vitro, as investigated by the co-applicant's group at the MPI. The impact mutations in either FoxP2 or Tbr1 on this interaction is very well explained in: De novo TBR1 mutations in sporadic autism disrupt protein functions, 2014, Nature Communications, 5:4954
Cntnap2 is a gene in which mutations can lead to many language-related phenotypes. It has been implicated in autism and schizophrenia, but also dyslexia and other language disorders. Thus, Cntnap2 mutations can lead to phenotypes related to language disorders, validating our interest in this gene. Additionally, Cntnap interacts with FoxP2 is well, as FoxP2 can bind to intron 1 of Cntnap and regulate it's expression. The genetic evidence for Cntnap2 mutations and possible interactions with FoxP2 has been shown in research conducted by the co-applicant's group at the MPI. This research is shown in: Shining a light on CNTNAP2: complex functions to complex disorders, 2014, European Journal Of Human Genetics, 2:171-178
We conclude that these three genes are interesting because of two main features: First, data grom human patients indicates that mutations in these genes lead to disorders with a similar phenotype as mutations in FoxP2 (language related). Second, there is convincing in vitro evidence for functional interaction between these genes and FoxP2. Any other gene that we could become interested in will need to confirm to these guidelines in order to be considered for further investigations in mice.
We will investigate mutations in these genes using genetically modified mouse models that mirror the genotype found in affected humans. We expect our initial results to show specific brain regions or cells to be affected. This will enable us to use transgenic strategies to create modifications to specific these regions (e.g. Cre-mediated cortex-specific transgenes) or cell populations (e.g. Dopamine-D1 receptor expressing neurons).

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

Combining the expertise of [redacted] I as well as the [redacted] we will be able to investigate the proposed research directions in a multidisciplinary manner using state-of-the-art techniques. Furthermore, the facilities required to conduct our research, [redacted] in Nijmegen at the animal facility (Centraal Dierenlaboratorium, CDL) of the Radboudumc. In addition, the [redacted] group's state-of-the art electrophysiology laboratory will allow very detailed investigations of the cellular and physiological properties at the single-cell level.
In summary, this research plan aims to investigate the effect of mutations in language-related genes on a molecular, cellular, neurophysiological and behavioral level. This will enable us to devise a new approach for identification, investigation and possible remediation of disorders affecting motor regions governing speech and language tasks.

3.3 Relevance

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

Increased knowledge regarding the influence of genetic mutations related to speech and language on the brain is necessary to further our understanding of speech related disorders, which have a very large societal impact.
However, there are specific disciplines within neuroscientific research, especially on the synaptic and circuitry level, which have only sparsely been explored regarding speech and language disorders. Our previous collaborative efforts have shown tentative evidence for the effect of Foxp2 on a circuitry level in specific brain areas. We want to continue our investigation of these defined brain regions, especially during developmentally relevant time periods, using new techniques to study how connections within and between brain regions are affected. We aim to investigate the impact of mutations in Foxp2, and the genes that interact with it, on neuronal function in working mouse brains. We will specifically focus on brain regions shown to be affected by FOXP2 mutations in humans. This will enable us to extend the rich body of previous and ongoing molecular work in cell models into brain circuitry and function, and thus fill specific gaps in our knowledge with regard to speech and language disorders.
Overall, we are convinced that this research will increase our knowledge with regard to speech and language disorders. We will be able to increase knowledge on the specific brain areas, cell populations and genetic mechanisms that are affected by mutations in language-related genes. These mutations have a large impact on the overall function and wellbeing of affected persons, as speech and language impairments greatly impact everyday life. Better characterization and understanding of these disorders will result in a mechanistic characterization of a common neural pathways underlying different monogenetic speech disorders. This will be the first time such a pathway has been completely characterized on all levels from genetic disorder to motor phenotype. Combining the existing knowledge in our groups on both genetics and cellular physiology, we will be able to unravel specific cellular deficits for genetic mutations related to speech and language disorders.
Using the knowledge gained during this 5 year project, we will be able to explain motor deficits affecting speech production from a cellular, developmental and circuit perspective. This is not necessarily limited to language disorders, as the investigated brain regions govern other motor

tasks as well. Exact knowledge of the proteins, cells, and brain circuitries affected in language-related disorders will enable a much more detailed diagnosis and development of circuitry-level based therapeutic interventions.

3.4 Research Strategy

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

Previous research has uncovered possible interactions between language related genes, most notably Foxp2, and other genes. For example, [redacted] [redacted] group (Vernes et al. 2011) reported on the interaction between Foxp2 and a large number of genes important for neurogenesis, neuronal outgrowth and synaptic activity. This research was done both in cell models and primary neural cultures of Foxp2 mouse models, and strongly implicated Foxp2 function in neuron-related gene networks.

Primary neuronal cultures, which form an integral part of our research, enable us to study connectivity in a very controlled environment. Neurons function normally and we can use these primary cultures to measure many different cellular properties and cell specific effects. However, these cultures do not conserve the circuitry normally found in the brain, especially complicated networks such as cortical layering or projections to different brain regions. Thus, in addition to using our established primary culture protocol, we aim to investigate network properties of intact mouse brains as well.

Using a multitude of experimental techniques, we aim to investigate the influence of language-associated genes on brain development and function on several levels: Molecular, cellular, physiological, and behavioural.

- Molecular: Currently, Foxp2 is the gene with the strongest link to language phenotype. Tentative evidence has been found linking other genes to language related phenotypes as well, and we would like to include these and any other newly discovered genes as possible genes of interest in this research proposal. Foxp2 is a transcription factor, which are genes that can activate or suppress the expression of other genes and thus act as "switches" in the genetic programming of cells. The actual effects on the neurons expressing those genes are thus likely to be indirect. Gene networks have implicated Foxp2 in neurite outgrowth, which was corroborated in subsequent in vitro experiments in primary neuronal cultures. Continuing those with more detail still forms an important part of our project.

However, while cell cultures are well-suited to investigate the molecular effects on single cells, these *in vitro* methods are not well-suited to study the intricate, interconnected mechanisms at work in the developing brain. Therefore, the developmental aspects many of the possible effects of Foxp2 mutations, especially on neurogenesis and neuronal localization, need to be validated *ex vivo* in brain slices. This research objective will be achieved by stainings *ex vivo* preparations of transgenic mouse brains with in-situ hybridizations (ISH, for gene expression) and immunohistochemistry (IHC, for protein levels). Using in-situ hybridizations will enable us to investigate gene expression on the brain-region and cellular scale: Confirm the presence of mRNA for Foxp2 and other language-related genes in different brain regions and stages of neuronal development, from mid-gestational embryonal stages to early postnatal stages. This technique is especially well-suited because it allows precise quantification of mRNA production, and thus gives a measure of gene activation in the context of the developing brain.

Additional steps of regulation happen in the *translation,* the production of proteins from mRNA. Therefore, in order to localize and quantify the final activity of our genes of interest, we also need to measure the amount of proteins that are produced. This research objective is best achieved by immunostainings, which allow to precisely measure and localize protein expression in the tissue of interest. Using this method we can investigate the impact of mutations in language-related genes on translation from mRNA into functional protein. Furthermore, combining the outcomes of cell cultures, ISH and IHC enables us to precisely quantify regulation of gene activity by our genes of interest at all relevant levels. These outcome

measures together will enable us to form a strong hypothesis on the impact mutations in language-related genes have on mRNA and protein levels of Foxp2 in different brain regions during development.
- Cellular: It is a well-established paradigm that numerous genes are expressed in specific cell populations in the brain, at specific developmental timepoints. This is the case for Foxp2 as well, whose temporal and spatial expression pattern is deemed crucial for proper brain function.
However, the exact mechanisms affected by mutations in Foxp2, and other genes which affect speech and language are not well characterized during development. This aim focuses on cellular characterization during development, especially regarding network establishment (anatomical connectivity), and network function (synaptic connectivity). Foxp2 has already shown to be important for establishment of proper cellular growth in primary cultures; However, the developmental profile still remains to be assessed in detail. For other language-related genetic mutations, we would like to be able to continue our investigation after initial validation in primary cultures as well.
To assess the importance of transcription factors such as Foxp2 during development, we aim to extract genetically modified and wild-type embryos at mid-to-late gestational stages, when the relevant brain development takes place. We will focus on processes such as neurogenesis, neuronal migration, neurite growth, network establishment and pruning. Effects on these processes will be investigated by comparing expression levels of relevant genes by staining (ISH and IHC).
Since different neuronal populations are generated at different developmental time points (e.g. in the generation of the cortex), neurons can be “birthdated” by injection of a labelling substance for specific neuron types (e.g. BrdU) during embryonal development. In order to investigate network establishment, we aim to trace connections between brain regions using *in vivo* injections of anatomical tracers into the brains of adolescent / adult animals. Tracers are substances that label neurons around the injection site, visualizing the up- and downstream projection targets of those neurons. This will enable us to investigate the effects of Foxp2 on network establishment and function during later stages of development as well as during adult life as functional Foxp2 expression is present during adolescence and adulthood as well. We aim to enable the investigation the effect of language-related genes' effect on cell morphology and connections to other cells. This cannot be done by visualizing Foxp2 mRNA or protein, as these outcome parameters do not give us information about the intercellular effects of mutations in language-related genes. Visualizing the connections cells make when Foxp2 is mutated, and comparing these connections to connections in wildtype cells, will give us detailed information about the effect Foxp2 has on the establishment of proper connections in the brain. By establishing which connections in the brain are affected by mutations in language-related genes will give insights into the circuit-level defects that underlie the actual physiological and behavioural dysfunctions that characterize language disorders.
- Physiology:
With cellular and molecular research, we can identify and characterize the cell types and brain regions of importance on a genetic, morphological and anatomical level. This does however not explain how these changes affect cells on the functional level. In order to investigate this we will need to investigate cellular activity in a system where these cells are still functioning as they would in the living animals. To this end, electrophysiological measurements on acute *ex vivo* brain slice preparations are ideally suited: They allow for high-throughput measurements in a well-controlled environment while reducing the discomfort for the animals to a short anesthesia and euthanasia step. We aim to use electrophysiology on acute brain slices to answer how these genetic, morphological and anatomical changes affect cellular activity. When processed quickly, *ex vivo* preparations of brain slices retain viable neurons for the next 8-12 hours, allowing detailed investigations of the neuron’s electrophysiological properties. [redacted] has ample experience with electrophysiological measurements on the single cell (patch clamping) as well as multi-cell (multi-electrode array) level.
Using these experimental tools has given us initial insight into the effect of Foxp2 mutation on cell populations in the cortex and striatum during development. However, we would like to extend our research to other brain networks in addition to the striatum. Lastly, this experimental paradigm can be extended to investigating the effect of newly established language associated genes on cellular activity.

- Behaviour/Rescue: Lastly, we would like to be able to translate our findings back to known behavioural phenotypes. Using our results from the previously described research lines, we can investigate in depth the effects of the mutations on behaviour. Previous research on the effect of Foxp2 mutation on motor performance has been on animals where Foxp2 is decreased in all cells and tissues. These paradigms can be vastly improved by having brain region- or even cell-type specific knockout mouse models for the relevant genes, which we propose to introduce into these experiments. Additionally, our mechanistic hypotheses can be directly proven by rescue experiments. The first gene specifically related to language, Foxp2, was first identified in humans showing a facial musculature specific motor deficit. Therefore, specific motor tasks are an appropriate tool to assess the effect of mutations in Foxp2 and other language-related genes in mouse models. Previous behavioural research on Foxp2 mutant mice, for example, showed an effect on motor coordination: In their 2008 article, Groszer et al. report impaired rotarod performance in Foxp2 mutant mice.
We aim to test the hypotheses generated by the previously mentioned objectives in an *in vivo* mouse model, using behavioural assays such as the accelerating rotarod. Animals are placed on a rotating beam on which they need to walk to prevent falling off. The time it takes for an animal to fall, as well as the speed with which an animal learns the task, can be used as measures for motor coordination and motor learning.
By manipulating cell populations with spatially or genetically targeted manipulations, for example targeting optogenetic receptors to specific subpopulations in the motor circuitry, we aim to prove the functional relevance of the targeted cell populations for the behavioural phenotype.
Coherence: Our experimental procedures are set up to follow each other logically. For example, results from e.g. molecular research (primary neuronal cultures) are necessary for us to form a correct experimental hypothesis regarding the cellular research (electrophysiology). Molecular research will be used to identify targets for cellular research. Defined cell populations can then be investigated using cellular techniques and electrophysiological tools. Finally, our behaviour and rescue experiments will depend on the cellular mechanisms found to be affected by mutation. This approach will be used several times over the course of the experiment to address different aspects of language-related gene function. As stated before, a large part of our research until now has focused on global Foxp2 mutation. We will extend this to mutation of Foxp2 in specific brain regions or cell populations to investigate the relative importance of Foxp2 in different parts of the brain's motor circuitry. Furthermore, we plan to apply the same analysis pipeline to mouse models of other candidate genes for language-related deficits. This combinatorial approach will serve to elucidate whether the different genetic mutations cause convergent defects in the brain's motor circuitry.
Go/no go moments: Between each part of our experimental protocol, we can assess the viability of continuation or the necessity to readdress our research strategy. This is especially relevant for designing new experiments which follow from previous results. For example, results obtained using primary neuronal cell cultures will aid in deciding on the appropriate electrophysiological experiments. In turn, if the electrophysiological experiments confirm the phenotype found in neuronal culture assays, the affected brain region will be assessed in tracing and behavioural experiments.
Next to designing new experiments, we need to assess the viability of the project when we will investigate newly found genetic mutations. Depending on the time left for the project upon discovery and implementation, a go/no go decision can be made to run a full / part of / no experimental procedure for this genetic mutation.
Lastly, we will regularly discuss the viability of each aspect of the project. As we have four experimental paradigms we will employ we will need to assess each separately as well as in light of the essential aim of the project. If we decide a certain paradigm is no longer suited or needed for us to reach our conclusions we will be able to be more efficient with regard to our experimental procedures.
Timeline: Our project proposal has different aspects which are interdependent. From the moment the license is approved until the end of the project animals will be kept in breeding. The research on two Foxp2 mutations (R552H and S321X) is already in a more advanced stage, and therefore substance administration and behavioural testing with animals affected by these mutations will already happen during the first 2 years of this project (see timeline). However, this project is not limited to Foxp2, and recently discovered language-related genes (e.g. Tbr1 or Cntnap2) will be investigated using the same experimental procedures. A full investigation will take approximately 2,5-3 years from the start of the project (see timeline), and therefore the final addition of new genetic lines to this project application could happen around 01-2018. This strategy is outlined in two tables. The first table gives an overview of specific timepoints when new investigations can be started, especially for behaviour. The second table

shows a detailed outline from start to finish for an example gene, going from the first characterization (after in vitro assessment) to the final experiments for one specific mutation.

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

The experimental components mentioned above are going to be realised in several different animal procedures, as described in the following appendices:
- Stock breeding without discomfort and organ extraction (Appendix1): Breeding and maintenance of all animal strains that do not suffer discomfort as a result of their genetic condition. Animals may be euthanized at defined timepoints for organ harvesting, typically the brain or embryos of a defined developmental stage. Alternatively, they may be transferred to separate experiments for substance administration (Appendix 3, see below) or behavioural testing (Appendix 4, see below).
- Breeding with discomfort and organ extraction (Appendix 2): This is a separate breeding procedure to accommodate breedings with a risk of discomfort due to the genetic condition. At the moment, this is only the case for heterozygote - heterozygote crossings of our Foxp2-mutant lines: Around 25% of the offspring, namely homozygous mutant animals, are going to experience moderate distress throughout their life and usually die around postnatal day 30. Animals may be euthanized for brain harvesting, or transferred to separate experiments for substance administration (Appendix 3, see below) or behavioural testing (Appendix 4, see below). When we acquire new animal strains, we will discuss with the IvD if animals should be allocated to breeding with or without discomfort depending on the genotype.
The large majority of experiments will be done with *ex vivo* brain / embryonic brain preparations, which means that almost all animals are only going to experience mild, momentary discomfort from timed breedings and euthanasia. Only a very small portion of animals will experience additional discomfort due to the experiments, as outlined below.
- Substance Administration and organ extraction (Appendix 3): Animals will receive either globally acting substances, or specific targeted injections. Examples of globally acting substances include inhibiting peptides, psychoactive drugs, or labelling substances for “birthdating” newly generated neurons. These substances can be administered via injection or oral gavage. This approach is the preferred administration approach, and is essential to investigate the effect of genetic mutations on brain development and neuron localization.
Alternatively, animals receive targeted injections of locally-acting substances at stereotactically defined locations in the brain. Examples include viral vectors to drive gene expression, or tracers to visualize neuronal connections. This approach requires a survival surgery, during which the injection is placed through a small craniotomy. Contrary to the globally-acting administrations described above, this approach allows the specific modification of narrowly defined cell populations or brain regions, allowing us to verify and extend previous findings from single-cell electrophysiology in *ex vivo* brain slices.
In both cases, the animal will need to survive for up to 3 weeks after the end of the treatment to allow for the effects to fully develop. Subsequently,

the animals will be either euthanized and their brains harvested, or transferred to behavioural testing (Appendix 4, see below) to assess the effect of the manipulation.
Both administration methods will be used in multiple ways, either to visualize cellular properties, or to perform rescue experiments. These rescue experiments are based on previous data implicating certain cellular mechanisms in the genesis of the mutant phenotype. Targeted interference to restore circuit functions to wildtype levels is considered the final proof that the system at hand has been understood sufficiently well, and also lends additional translational value to future applications in the clinic.
- Behavioural testing (Appendix 4): Animals will be tested on their motor learning skills and cognitive function. The experiments form a crucial translational link between our genetic and cellular evidence and a behavioural phenotype. In addition, these behavioural paradigms are well-suited to assess rescue experiments: They provide an insight into the degree of influence specific interventions can have towards restoring performance back to wild-type levels.
Here you will find an extended table describing each suggested animal procedure and the animal numbers we have calculated as necessary to properly investigated our experimental research questions.

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

The described components all serve the goal stated above, namely to unravel the specific mechanisms of action for genes found in human speech and language disorders. In essence, the gene mutation (genotype) and human speech disorder (phenotype) are known, and we strive to find the specific mechanisms that act in between. To this end, we need to know the mechanisms acting on the molecular, cellular, network and behavioural level. We will use genetically modified mice to investigate effects of specific genes in all these research areas. Results obtained from experiments on one level of complexity (i.e. cellular) will help to form hypotheses in the others (i.e. behavioural). There is sufficient previous research to allow testable hypotheses with regard to each part of this project license. Thus, we are convinced that this project is coherent and focused enough to warrant a project license.
Lastly, we are confident we will be able to utilize the 5 years given in this project license to design and perform experiments aimed to localize, characterize and rescue deficits in the neural motor pathway caused by genes shown to be involved in speech and language development.

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

Serial number	Type of animal procedure
1	Stock breeding without discomfort and organ extraction
2	Breeding with discomfort and organ extraction
3	Substance administration and organ extraction
4	Behavioral testing and organ extraction

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	10300	
1.2	Provide the name of the licenced establishment.	Stichting Katholieke Universiteit Nijmegen	
1.3	List the different types of animal procedures. Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.	Serial number 1	Type of animal procedure Stock breeding without discomfort and organ extraction

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.

Primary goal:
The primary goal of this research project is to unravel the function of language-related genes in the motor system of the mammalian brain. We aim to investigate the function of those genes on the molecular, cellular, physiological and behavioural level. The first three aspects (molecular, cellular and physiological) will mainly be addressed by using *ex vivo* preparations of brain tissue from genetically modified mice at embryonal, adolescent and adult life stages.
General Design:
The aim of this animal procedure is to breed and maintain sufficient numbers of mice genetically modified to have specific defects in language-related genes such as Foxp2. A small part of the resulting mice will be used in substance administration (Appendix 3) and behavioural experiments (Appendix 4). Furthermore, it covers the extraction of brains for *ex vivo* experiments: Generally, animals will be bred and maintained to experimentally appropriate timepoints, then anesthetized and sacrificed for the extraction of organs, typically the brain and/or embryos. This procedure ensures minimal discomfort for the animals, while allowing extended studies on the *ex vivo* preparations.
Ex vivo preparations are ideally suited to investigate the molecular, cellular and physiological aspects of language-related gene function, because the preparations allow for a much better control of environmental parameters than would be possible *in vivo*. In addition, using embryonal brains as a source of primary neurons for cell cultures allows studying the molecular and cellular properties with excellent specificity, while greatly reducing the amount of animals required: Brain preparations from one litter of embryos results in millions of cells that can be cultured and studied in great detail in experiments lasting up to several weeks.
This procedure is covers the animals suffering no discomfort from their genetic modifications. Most of the strains we currently use under multiple research projects do breed and develop normally. Breedings with strains that could, for a portion of their offspring, lead to discomfort, are outlined in a separate appendix (see Appendix 2 “Breeding with discomfort”). However, the large majority of breedings is going to be discomfort-free, and therefore covered by this appendix.

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

Animals will be group housed and mated according to well established housing procedures for animal breeding. To achieve the best possible protection from contamination, the animals will be kept in a barrier environment such as individually ventilated cages (IVCs), fed sterilized chow food, and only handled under a flow hood.
Animals will be genotyped at the appropriate postnatal age, via minimally invasive techniques such as ear biopsies, which induce only momentary discomfort. This technique has the additional advantage of allowing individual recognition of the animals.

Depending on the experimental setup, the animals will be used for substance administration experiments (covered in Appendix 3), behavioural testing (covered in Appendix 4), or organ extraction for *ex vivo* preparations (covered in this appendix).
Oestrus cycle measurement for timed mating (mild, 5 minutes, several times): In order to get precisely timed pregnancies for embryonal extraction at precisely defined timepoints, the oestrus cycle of the females destined for timed breedings will be monitored. After the timed mating, the cage will be visually inspected for the presence of a vaginal plug, since this indicates that a mating has actually taken place. The combination of the two procedures allows for very precise control of the mating timepoint. This, in turn, allows for precise timing of the embryonal age, which is crucial to the cortical development studies we plan to undertake. Depending on the breeding schedule, one or two females might be set with a single male. After the mating, the male will be removed from the cage and housed solitarily, because breeding males do tend to fight with when housed with other males after breeding.
Terminal anaesthesia, sacrifice and organ extraction (non-recovery, <1 minute, once)
a) Embryo extraction: The pregnant mothers will be anesthetized with an anaesthetic such as pentobarbital, then quickly sacrificed. The embryos will be extracted to prepare *ex vivo* brain material for use in stainings, cell culture, or electrophysiological experiments. This procedure keeps discomfort to the pregnant mother to a minimum, and the use of embryonal brains (especially for primary neuronal cultures) allows us to make very efficient use of the sacrificed animals, as millions of cells can be extracted and cultured for *in vivo* experiments. To add additional use per sacrificed animal, we will consider in our experimental plans to concurrently extract the pregnant mother's brain along with the embryos.
b) Brain extraction at adolescent-adult timepoints: The animal will be anesthetized via an anaesthetic such as pentobarbital, then quickly sacrificed. Subsequently, the brain will be quickly prepared out of the skull and immediately used for further experiments on the molecular and cellular levels. This approach ensures that the animals suffer as little discomfort as possible, while allowing us to measure gene expression in various parts of the brain's motor pathways. The use of *ex vivo* preparations allows for very precise localization and quantification of gene expression and circuit layout that would not be possible in any other way.
Special case electrophysiology: Transport to the lab (mild, 5 min, once), followed by terminal anaesthesia, decapitation and organ extraction (non-recovery, <1 minute, once): To fully understand the effects of language-related gene defects on the physiological level, i.e. the function of the brain's motor circuits, we aim to measure the electrophysiological properties of single cells and cell networks in acute brain slices of adolescent and adult mice. If done correctly, freshly extracted brain tissue can be cut into acute slices, in which most neurons keep living and functioning for up to 12 hours. During this time, the electrical activity of those neurons, and the networks they form, can then be probed in our specially equipped electrophysiology laboratory. The electrophysiological portion of the experiments necessitates transport of the live animal into our specially equipped electrophysiology laboratory, which is located in the same building complex as the CDL. The transport to our specially equipped electrophysiology lab is absolutely essential, since the technique requires immediate processing of the brain (< 1 minute after decapitation) in order to yield viable material. After processing, the tissue is extremely sensitive to movement, making any further transportation impractical. The animals will only experience mild discomfort during transport to the laboratory, which happens indoors and lasts for about 5 minutes. Once in the laboratory, they will be immediately anesthetized and decapitated, keeping discomfort to the animals to a minimum. This approach allows us to study the neurons in a well-controlled environment, and in much greater detail and with higher yield than would be possible *in vivo*.

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

For this breeding procedure, genetically modified mice of different strains will frequently be crossed with each other. Where necessary, we will breed

heterozygotes of the two strains with each other, and select the resulting offspring based on genotype. Alternatively, we will cross heterozygotes with wild-type mice.
The maintenance of the genetically modified lines for stock purposes will be kept to the necessary minimum, i.e. usually one breeding trio and their most recent offspring per strain. We strive to maximise utilisation of sacrificed animals: For example, when a timed-mated mother mouse is sacrificed for embryo extraction, the concurrent extraction of the mother’s brain can provide an additional (adult) data point. To ensure compatibility and minimise backcrossing, mice will be maintained on a single genetic background (C57/BL6).
In order to estimate the animal numbers required for experiments, we will use the program G*power (http://www.gpower.hhu.de/), assuming a clear and consistent change (effect size = 0.8), a power of 0.8 and a significance level of 0.05.

B. The animals

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

Species:
All experiments will be performed on genetically modified mice. Since the general goal of the project is to research the language-related gene Foxp2 and its interaction partners, we plan to use:
- Mice with genetic modifications in the Foxp2 gene
- Mice with genetic modifications in other Foxp family member genes
- Mice with genetic modifications in genes whose human orthologues are relevant for neurodevelopmental disorders
- Mice with genetic modifications to express transgenes such as LacZ, eGFP and Cre in a restricted manner: For example specific cell types or brain areas, or only at certain developmental timepoints.

Mice from those groups will frequently be intercrossed as experiments necessitate. Furthermore, the mouse is an ideal model organism for this type of research, because it is a mammal and therefore phylogenetically closely related to humans, yet also uniquely amendable to genetic modifications. Mice share large genetic similarity with humans, and their brain's motor control circuitry has also been shown to be very similar. Taken together, these factors mean that mice are the model of choice for our project to completely characterize the influence of language-related gene defects from the molecular to the behavioural level.
Origin:
The mice will be sourced from recognized commercial breeders, collaborators (following quarantine), and own breeding stock already successfully established at the CDL in Nijmegen.
Estimated numbers:
From previous experience, and with an eye on the number of mouse strains we will have to maintain, we estimate to need 5680 animals for this procedure over the next five years, 43% of the project's total animal number of 13280. This is due to the fact that mice from this breeding procedure provide the majority of animals for tissue extractions for both embryonal and postnatal timepoints. It should be noted that none of the strains used in this appendix suffers discomfort related to their genotype.
In addition, a subgroup of the animals for the experiments described in Appendices 3 and 4 is going to be derived from the same stock as animals for this appendix (Appendix 3: 1360 animals, Appendix 4: 720 animals). Since these animals are merely going to be bred under Appendix 1, and experimentally manipulated under Appendices 3 and 4, they are not counted towards the total of this Appendix (App. 1), but rather under Appendices 3 and 4.

Life stages:
Breeding: Since this is a breeding procedure, the animals will be maintained as a breeding colony: Animals of the appropriate genotypes will be kept until adulthood, and used for breedings until max. 300 days of age. Animals destined for experiments will be used at experimentally relevant timepoints (postnatal day 8 – adulthood).
Embryo extraction: Embryos will be extracted from pregnant mothers (P 60-300) at mid-late gestational ages (E11-E19.5). Since our genes of interest likely serve as high-level regulators during brain development, we plan to assess brain development in our mouse models, which requires extraction of embryonic brains at mid-late gestational periods. Furthermore, the neurons of mid-late gestational embryos are exceptionally well suited for use in cell cultures, allowing for tightly controlled experiments with minimal animal usage.
Postnatal brain extractions: We chose adolescent and adult timepoints because they provide optimal insight into the clinically relevant states of the mutant brain: Adolescence in mice roughly maps onto the first postnatal years in humans, which allows detailed research into the initial set-up of the motor system that is crucial for language in humans. Measuring in adulthood, on the other hand, is crucial to investigate the long-term effects of language-related gene defects.

Species	Origin	Maximum number of animals	Life stage
Mouse (C57BL6J) with genetic modifications in the Foxp2 gene	Own breeding, collaborators	1900	Embryonal - Adult (P<300)
Mouse (C57BL6J) with genetic modifications whose human orthologues are relevant for neurodevelopmental disorders	Collaborators	1520	Embryonal - Adult (P<300)
Mouse (C57BL6J) with genetic modifications in other Foxp family member genes	Recognized breeders, collaborators	1900	Embryonal - Adult (P<300)
Mouse (C57BL6J) with genetic modifications to express transgenes (LacZ, eGFP, Cre)	Own breeding, Recognized breeders, Collaborators	360	Embryonal - Adult (P<300)

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: The experiments build on a large volume of research by the involved research groups, mainly in cell cultures. Those cell cultures include immortalized cell lines, such as HEK293 or SHS5Y5 cells, which do not require the sacrifice of animals at all, and primary neuronal cultures, which are a very efficient use of extracted embryonal neurons. Immortalized cell lines are well-suited to research into basic molecular and genetic effects of our genes of interest, but do not form any neuronal connections. Neuronal cultures form random connections while growing *in vitro,* and thus are well-suited to assess general synaptic phenotypes at the single-cell level. However, neuronal cultures are not as well-suited to model complex processes that involve multitudes of different cell types. Our research questions also concern complex, interdependent processes such as brain development, wiring, or circuit function, all of which also require studying intact mouse brains. Thus, the single-cell perspective we gain from cell cultures will form the basis on which we formulate our hypotheses to test on the circuit level in *ex vivo* brain preparations from mouse models.
Reduction:
- The animal strains will be carefully chosen with regards to the current needs of the experiments. For example, we reduce the variability in our samples by measuring with littermate controls wherever possible. This allows us to reach stronger effect sizes with smaller groups of animals due to the lowered variability.
-Furthermore, the use of primary neuronal cultures from embryonic neurons greatly reduces the number of animals required to answer questions on the molecular and cellular aspects of our study: Brains from one litter of embryos allow for the extraction of millions of primary neurons, which can be cultured and serve for the basis of multiple *in vitro* experiments lasting several weeks.
Refinement:
-To guarantee the best possible environment and minimize chances of contamination for the animals, they will be housed in sterilized individually ventilated cages (IVCs), receive sterilized food and drinking water, and only be handled under a flow hood. This housing regime is considerably more sophisticated than the filter top cages commonly used for similar experiments, and adds additional layers of isolation from potentially interfering outside influences, such as viral infections.
- In addition, the use of anaesthesia in all terminal procedures actively minimizes animal discomfort. The use of the volatile anaesthetic isoflurane before electrophysiology experiments additionally ensures that the resulting brain slices, in which neurons live and function for several hours after preparation, are not influenced by anaesthetics residues.

- The timed breeding required for embryonal timepoints will be done only after ensuring optimal fertility (via oestrus cycle measurement) and checking for mating afterwards (via vaginal plug inspection), thus ensuring that the animals are only bred when chances of conception are optimal.
- The intercrossing of "marker" lines expressing fluorescent proteins in specific neuronal subpopulations in the brain will allow direct identification of experimentally relevant cell populations in e.g. electrophysiological experiments. This adds an additional level of detail to our research (cell population-specificity instead of brain region-specificity), without any additional discomfort to the animal. Leading theories in the field are proposing the clinical relevance of neuronal subpopulation-specific dysfunction, which makes this labelling approach all the more relevant.

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

1) Animals will be kept in a strictly isolated environment (IVCs, see above) to minimise chances of contamination. Furthermore, this procedure describes breedings only of lines with overtly normal phenotypes, i.e. that do not suffer from their genetic modifications. Animals will be monitored at least weekly, and any animal showing signs of any distress will be processed according to the humane endpoints outlined in section J.

Repetition and Duplication

E. Repetition

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

Accommodation and care

F. Accommodation and care

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

[X] No

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

G. Location where the animals procedures are performed

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

[X] No > Continue with question H.
[] Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

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

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

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

I. Other aspects compromising the welfare of the animals

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

We do not expect any adverse effects from the genotypes or housing, since the mice develop and breed normal and live without overt signs of discomfort. Nevertheless, animals will be closely monitored to detect any sign of undue distress.

Explain why these effects may emerge.

Despite the best countermeasures, some animals might develop illnesses, or experience social stress due to fighting among cagemates.

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

If the animal shows bodily or behavioural symptoms indicating undue distress, it will be euthanized according to the humane endpoints outlined below.

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.

Despite the best countermeasures, animals might sometimes show signs of undue distress. Outward signs such as a ruffled fur coat or wounds, and behavioural signs such as limping, hunched back, or immobility will be taken as sign for undue distress, and the animal will be sedated and euthanized immediately.

Indicate the likely incidence.

The likely incidence is very low, since the mouse strains are overtly normal in health and behaviour, and are housed in specially isolated individually ventilated cages, reducing outside exposure.

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).

Marking / tissue sampling for genotyping (mild, once, 100% of all animals): After the first postnatal week, the animals will be marked by an appropriate method such as ear biopsies, which induce only momentary discomfort. The resulting samples will be used for genotyping. In rare instances, due to technical failures in genotyping, a second sample may need to be taken.

Timed breedings (mild, few minutes, several times; 1600 females, 28% of totally 5680 animals for this Appendix): Precise timing of conceptions in timed breedings will be ensured, where necessary, by appropriate methods such as oestrus cycle measurement.
Euthanasia and organ extraction: The animals are going to be euthanized in one of the two following ways:
In case of electrophysiology: Transport to the lab (mild, 5 minutes, once; 2800 animals, 49% of totally 5680 for this Appendix). On the day of the experiment, animals will be placed in cardboard boxes with bedding material provided by the CDL staff. They will be quickly transported to our specially equipped electrophysiology lab in the Radboudumc, which is within the same building complex as the animal laboratory. The only discomfort the animals are going to experience during this time is movement and unfamiliar surroundings. Once in the laboratory, the animal will directly be euthanized and have their brains extracted as outlined below.
Euthanasia for tissue extraction (non-recovery, <1 minute, once; 2880 animals, 51% of totally 5680 animals for this appendix. Of these, the 1600 animals that are used for embryo extraction have undergone timed breeding procedures as described above): The animal will be sedated with an appropriate sedative such as isoflurane (in case of electrophysiology) or pentobarbital (for all other applications), and the absence of pain reception will be tested via e.g. forepaw pinching (absence of a reflex indicates total absence of pain sensation). Animals will be killed via cervical dislocation, decapitation, or transcardial perfusion, as the experiment necessitates. Typically, the brain will be extracted. In some cases, the brain will be extracted along with embryos of a defined developmental age.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

If the animals are not going to be used in other procedures first, they will be either maintained for stock breeding purposes or sacrificed for tissue extraction. The latter entails extracting brains from postnatal animals (adolescence – adulthood) for various measurement methods such as electrophysiology or stainings. Additionally, developmental aspects will be studied mainly in embryos of mid-late embryonal stage, which requires sacrifice of the pregnant mother. Extracted embryos will be used directly in experiments (e.g. stainings), or as a source of primary neurons for cell culture.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

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

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.

Primary goal:
The primary goal of this research project is to unravel the function of language-related genes in the motor system of the mammalian brain. We aim to investigate the function of those genes on the molecular, cellular, physiological and behavioural level. The first three aspects (molecular, cellular and physiological) will mainly be addressed by using *ex vivo* preparations of brain tissue from genetically modified mice at embryonal, adolescent and adult life stages.

General Design:
The aim of this procedure is to breed sufficient numbers of mice for planned experiments on the molecular, cellular, physiological and behavioural levels. This animal procedure covers a part of the breedings for experiments in all four aspects, namely breeding of animals with a chance of discomfort from their genetic modifications. The large majority of the animals is going to be used directly in experiments for *ex vivo* preparations at embryonal, adolescent and (in case of heterozygotes and wild-type mice) adult timepoints. A small minority will be used in substance administration (Appendix 3) and behavioural (Appendix 4) experiments. Currently, the only animals with a chance of genotype-related discomfort are homozygous offspring of heterozygote - heterozygote crossings for two of our mutant mouse lines: Foxp2-R552H and Foxp2-S321X mutants. Additional transgenes might be bred into the line if they provide no additional discomfort (e.g. GFP-expression constructs that visualize a subpopulation of neurons without further interfering with brain function). Thus, even though all heterozygote - heterozygote breedings of all Foxp2-mutant strains will be registered under this appendix, only 25% of the registered animals (homozygous mutant offspring) will actually experience discomfort. Those homozygote mutants experience reduced growth, impaired motor coordination, periods of akinesia, and die before reaching reproductive age. Thus, the homozygote mutants will not be kept for stock breeding, but rather be used in experiments or (in the rare case they are not used in experiments) get euthanized before P30 (see section J, Humane Endpoints). Since the homozygous Foxp2-mutant mice represent the strongest phenotype, they are crucial to some of our experimental outcomes, as the stronger phenotype allows us to find differences in a small number of animals that might otherwise only be detected with many more heterozygous animals. Furthermore, a large part of the research will be primary neuron cultures, which means that most heterozygous mutants will already be extracted at embryonal timepoints, and consequentially not suffer any discomfort.

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

Animals will be group housed and mated according well established experimental housing procedures for animal breeding. To achieve the best protection from possible contamination, the animals will be kept in a barrier environment such as individually ventilated cages (IVCs), fed sterilized chow food, and only handled under a flow hood.

Since this appendix specifically considers the breeding of animals with discomfort, appropriate additional measures will be taken to minimise the discomfort of those animals. For example, the only genotypes that currently would fall under this appendix have reduced movement coordination and stunted growth, and thus will receive food in mashed form and water in gel form if they struggle to reach the food hopper.
Animals will be genotyped at the appropriate postnatal age, via minimally invasive techniques such as ear biopsies, which induce only momentary discomfort. This technique has the additional advantage of allowing individual recognition of the animals.
Oestrus cycle measurement for timed mating (mild, 5 minutes, several times): In order to get precisely timed pregnancies for embryonal extraction at precisely defined timepoints, the oestrus cycle of the females destined for timed breedings will be monitored. After the timed mating, the cage will be visually inspected for the presence of a vaginal plug, since this indicates that a mating has actually taken place. The combination of the two procedures allows for very precise control of the mating timepoint. This, in turn, allows for precise timing of the embryonal age, which is crucial to the cortical development studies we plan to undertake. Depending on the breeding schedule, one or two females might be set with a single male. After the mating, the male will be removed from the cage and housed solitarily, because breeding males do tend to fight with when housed with other males after breeding.
Depending on the experimental setup, the animals will be used for substance administration experiments (covered in Appendix 3), behavioural testing (covered in Appendix 4), or organ extraction for *ex vivo* preparations (covered in this appendix).
Terminal anaesthesia, sacrifice and organ extraction (non-recovery, <1 minute, once)
a) Embryo extraction: The pregnant mothers will be anesthetized with an anaesthetic such as pentobarbital, then quickly sacrificed. The embryos will be extracted to prepare *ex vivo* brain material for use in stainings, cell culture, or electrophysiological experiments. This procedure keeps discomfort to the pregnant mother to a minimum, and the use of embryonal brains (especially for primary neuronal cultures) allows us to make very efficient use of the sacrificed animals, as millions of cells can be extracted and cultured for *in vivo* experiments. To add additional use per sacrificed animal, we will consider in our experimental plans to concurrently extract the pregnant mother’s brain along with the embryos.
b) Brain extraction at adolescent-adult timepoints: The animal will be anesthetized via an anaesthetic such as pentobarbital, then quickly sacrificed. Subsequently, the brain will be quickly prepared out of the skull and immediately used for further experiments on the molecular and cellular levels. This approach ensures that the animals suffer as little discomfort as possible, while allowing us to measure gene expression in various parts of the brain’s motor pathways. The use of *ex vivo* preparations allows for very precise localization and quantification of gene expression and circuit layout that would not be possible in any other way.
Special case electrophysiology: Transport to the lab (mild, 5 min, once), followed by terminal anaesthesia, decapitation and organ extraction (non-recovery, <1 minute, once): To fully understand the effects of language-related gene defects on the physiological level, i.e. the function of the brain’s motor circuits, we aim to measure the electrophysiological properties of single cells and cell networks in acute brain slices of adolescent and adult mice. If done correctly, freshly extracted brain tissue can be cut into acute slices, in which most neurons keep living and functioning for up to 12 hours. During this time, the electrical activity of those neurons, and the networks they form, can then be probed in our specially equipped electrophysiology laboratory. The electrophysiological portion of the experiments necessitates transport of the live animal into our specially equipped electrophysiology laboratory, which is located in the same building complex as the CDL. The transport to our specially equipped electrophysiology lab is absolutely essential, since the technique requires immediate processing of the brain (< 1 minute after decapitation) in order to yield viable material. After processing, the tissue is extremely sensitive to movement, making any further transportation impractical. The animals will only experience mild discomfort during transport to the laboratory, which happens indoors and lasts for about 5 minutes. Once in the laboratory, they will be immediately anesthetized and decapitated, keeping discomfort to the animals to a minimum. This approach allows us to study the neurons in a well-controlled environment, and in much greater detail and with higher yield than would be possible *in vivo*.

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

The maintenance of the genetically modified lines for stock purposes will be kept to the necessary minimum, i.e. usually one breeding trio per line and their most recent offspring. Often, as in timed-breeding for embryonal timepoints, the concurrent extraction of the sacrificed mother's brain will provide an additional data point. To ensure compatibility and prevent backcrossing, all mice will be maintained on a single genetic background (C57/BL6).
In order to estimate the animal numbers required for experiments, we will use the program G*power (http://www.gpower.hhu.de/), assuming a clear and consistent change (effect size = 0.8), a power of 0.8 and a significance level of 0.05.

B. The animals

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

Species:
This appendix covers breeding of genetically modified mice with the possibility of offspring experiencing discomfort. Currently, this only is the case with heterozygote - heterozygote mutant Foxp2 R553H or S321X breedings, where homozygous offspring (ca. 25% of the litters) is known to suffer moderate discomfort. These crossings are crucial to this project, as they provide the strongest neuronal phenotype and thus enable us to investigate the affected circuitry with much less animals than would be possible if we only used heterozygous animals. Additional transgenic lines (e.g. GFP constructs) may be crossed with the Foxp2-mutant mice as experiments necessitate, provided that the resulting offspring suffers no additional discomfort from the transgenes. For example, we currently use GFP construct lines to label experimentally relevant neuron populations in the striatum, an important part of the brain's motor circuitry. Having specific neuron populations readily labelled is an invaluable tool for our experiments: It enables us to specifically target well-defined subpopulations without the need for additional discomfort for the animals, contrary to labelling with e.g. viral injections. Furthermore, the mouse is an ideal model organism for this type of research, because it is a mammal and therefore phylogenetically closely related to humans, yet also uniquely amendable to genetic modifications. Mice share large genetic similarity with humans, and their brain's motor control circuitry has also been shown to be very similar. Taken together, these factors mean that mice are the model of choice for our project to completely characterize the influence of language-related gene defects from the molecular to the behavioural level.

Origin:
The Foxp2-mutant lines described above are already well-established and successfully bred by both applicant groups under several successfully reviewed projects. Heterozygotes for breeding will be taken from our own stock, and bred according to well-established standard protocols. Their homozygous mutant offspring will be used for experiments typically early in life (before P21), because the homozygous mutants usually die before reaching reproductive age. Heterozygote and wild-type animals will be either used for experiments or euthanized. Heterozygotes may be maintained for further breeding under this procedure until adulthood (< postnatal day 300).
Estimated numbers:

From previous experience, we estimate to need 3400 animals for this procedure over the next five years. Of the total contingent of animals in this procedure, only ca. 20% (heterozygous mutants) will actually experience discomfort, and the remaining 80% will not suffer any discomfort from their genotype.
The numbers for heterozygotes are higher because those mice are also used as a source for embryos for primary neuronal cultures.Therefore, using comparatively few mice, we are able to support a major *in vivo* portion of the project, thus minimizing animal use.Breeding Foxp2-mutant mice in Het - Het crossings is indispensable for this project's objective, since the resulting homozygous mutant mice have the strongest brain-related phenotype, and thus allow us to probe more intricate mechanisms than would be possible with the heterozygotes alone.

Species	Origin	Maximum number of animals	Life stage
Mouse (C57BL6J) with homozygous Foxp2 mutation	Own breeding	800	Embryonal - early adulthood (P30)
Mouse (C57BL6J) with heterozygous Foxp2 mutation or Wildtype mouse	Own breeding	2600	Embryonal - adulthood (P<300)

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: The experiments build on a large volume of research by the involved research groups, mainly in cell cultures. Those cell cultures include immortalized cell lines, such as HEK293 or SHS5Y5 cells, which do not require the sacrifice of animals at all, and primary neuronal cultures,

which are a very efficient use of extracted embryonal neurons. Immortalized cell lines are well-suited to research into basic molecular and genetic effects of our genes of interest, but do not form any neuronal connections. Primary neuronal cultures form random connections while growing *in vitro,* and thus are well-suited to assess general synaptic phenotypes at the single-cell level. However, neuronal cultures are not as well-suited to model complex processes that involve multitudes of different cell types. Our research questions also concern complex, interdependent processes such as brain development, wiring, or circuit function, all of which also require studying intact mouse brains. Thus, the single-cell perspective we gain from cell cultures will form the basis on which we formulate our hypotheses to test on the circuit level in *ex vivo* brain preparations from mouse models.

Reduction:

-Furthermore, the use of primary neuronal cultures from embryonic neurons greatly reduces the number of animals required to answer questions on the molecular and cellular aspects of our study: Brains from one litter of embryos allow for the extraction of millions of primary neurons, which can be cultured and serve for the basis of multiple *in vitro* experiments lasting several weeks. This is specifically important in this procedure, since it means that a large volume of research on heterozygous mutant brains is taking place *in vitro* with prepared neurons from embryonal brains, thus greatly reducing the need for homozygous mutants to be born.

- The inclusion of homozygous mutants into our research plan allows us to investigate graded effects from unaffected (homozygous wild-type) to severely affected (homozygous mutant), thus allowing us to have multiple, interlinked controls within each litter. Furthermore, comparing heterozygous and homozygous mutant mice will allow to identify genetic compensation mechanisms likely at play, where related genes take over part of the function. Identifying those clinically relevant mechanisms would not be possible when only using heterozygous mutant mice.

Refinement:

-To guarantee the best possible environment and minimize chances of contamination for the animals, they will be housed in sterilized individually ventilated cages (IVCs), receive sterilized food and drinking water, and only be handled under a flow hood. This housing regime is considerably more sophisticated than the filter top cages commonly used for similar experiments, and adds additional layers of isolation from potentially interfering outside influences, such as viral infections.

- In addition, the use of anaesthesia in all terminal procedures actively minimizes animal discomfort. The use of the volatile anaesthetic isoflurane before electrophysiology experiments additionally ensures that the resulting brain slices, in which neurons live and function for several hours after preparation, are not influenced by anaesthetics residues.

- The timed breeding required for embryonal timepoints will be done only after ensuring optimal fertility (via oestrus cycle measurement) and checking for mating afterwards (via vaginal plug inspection), thus ensuring that the animals are only bred when chances of conception are optimal.

- The intercrossing of "marker" lines expressing fluorescent proteins in specific neuronal subpopulations in the brain will allow direct identification of experimentally relevant cell populations in e.g. electrophysiological experiments. This adds an additional level of detail to our research (cell population-specificity instead of brain region-specificity), without any additional discomfort to the animal. Leading theories in the field are proposing the clinical relevance of neuronal subpopulation-specific dysfunction, which makes this labelling approach all the more relevant.

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

1) Animals will be kept in a strictly isolated environment (IVCs, see above) to minimise chances of contamination. Animals will be monitored at least weekly, and any animal showing signs of any distress will be euthanized according to the humane endpoints outlined in section J.

Repetition and Duplication

E. Repetition

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

Accommodation and care

F. Accommodation and care

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

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

G. Location where the animals procedures are performed

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

[X] No > Continue with question H.
[] Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

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

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

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

I. Other aspects compromising the welfare of the animals

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

The animals maintained under this protocol are those where a chance on discomfort exists: 25% of the offspring of a Foxp2 mutant Het - Het crossing will be homozygous mutants and only those will be experiencing discomfort. Foxp2 homozygotes have a lower body weight than their littermates, difficulties with the righting reflex, reduced body weight and episodes of akinesia. Despite these abnormalities, pups appear to be cared for normally by the mother; For example, pups placed outside the nest area are retrieved. Homozygous Foxp2 mice die for unknown reasons around postnatal day 30, but histological analysis of the lung and haematocrit measurements suggest that death is not due to hypooxygenation caused by compromised lung function.

Explain why these effects may emerge.

The Foxp2 gene is a transcription factor, i.e. broadly regulates genes during embryonic development, for example in the lung, brain and spinal cord. Homozygous mutant animals (25% of animals in this procedure) apparently breathe normally, but have difficulty to engage their motor neuron pathways. These animals are the result of interbreeding Foxp2 mutant parents, and thus cannot be omitted from the experimental design.

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

As noted above, Foxp2 homozygous pups can be recognised by their phenotype soon after birth, being smaller in size and displaying a slower righting-reflex. In order to minimise the impact of homozygous Foxp2 disruption, pups will not be weaned from the mother, and food will be provided

on the cage floor in case they cannot reach the hopper. Furthermore, development will be closely monitored for especially severe courses of the developmental delay, in which the animal will be euthanized as outlined under Section J (Humane Endpoints, below).

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.

Despite the best countermeasures, animals might sometimes show signs of undue distress. Outward signs such as a ruffled fur coat or wounds, and behavioural signs such as limping, hunched back, or immobility will be taken as sign for undue distress, and the animal will be sedated and euthanized immediately.
Homozygous Foxp2 mutants:
All homozygous Foxp2 mutant animals not used for experiments will be euthanized at postnatal day P30. If at any earlier point in postnatal life they show a growth retardation of more than 50% compared to littermates (weight and/or size), they will be euthanized according to humane endpoints as well.

Indicate the likely incidence.

Of the animals described in this procedure, about 25% are going to be homozygous Foxp2 mutants that are known to suffer discomfort. The remaining 75% are heterozygous mutants or wild-type animals that do not suffer any adverse effects from their genotype. Thus, we expect the incidence of genotype-related discomfort to be around 25%. The likely incidence of discomfort from other causes is very low, as in other breedings without discomfort.

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).

Marking / tissue sampling for genotyping (mild, once, 100% of mice for this appendix): At some timepoint after the first postnatal week, the animals will be marked by an appropriate method such as earpunching, which only causes momentary discomfort. The resulting samples will be used for genotyping. In rare instances, due to technical failures in genotyping a second sample may need to be taken.
Timed breedings (mild, few minutes, several times 1000 females, 29% of a total of 3400 animals for this appendix): Precise timing of conceptions in timed breedings will be ensured, where necessary, by appropriate methods such as oestrus cycle measurement. Breeding males will be housed solitarily between timed breedings, as they tend to fight with other cagemates when placed back with other males.
Euthanasia and organ extraction: The animals will be euthanized for tissue extraction in one of the two ways described:

In case of electrophysiology: Transport to the lab (mild, 5 minutes, once, 1200 animals, 35% of total 3400 animals in this appendix). On the day of the experiment, animals will be placed in cardboard boxes with bedding material provided by the CDL staff. They will be quickly transported to our specially equipped electrophysiology lab in the Radboudumc, which is within the same building complex as the animal laboratory. The only discomfort the animals are going to experience during this time is movement and unfamiliar surroundings. Once in the laboratory, the animal will directly be euthanized and have their brains extracted as outlined below.
Euthanasia for tissue extraction (non-recovery, <1 minute, once, 2200 animals, 65% of total 3400 animals in this appendix, Of those, the 1000 animals for embryo extraction will have undergone timed breeding as described above): The animal will be sedated with an appropriate sedative such as isoflurane (in case of electrophysiology) or pentobarbital (for all other applications), and the absence of pain reception will be tested via e.g. forepaw pinching (absence of a reflex indicates total absence of pain sensation). Animals will be killed via cervical dislocation, decapitation, or transcardial perfusion, as the experiment necessitates. Typically, the brain will be extracted. In some cases, the brain will be extracted along with embryos of a defined developmental age.
For homozygous Foxp2-mutant mice (800, or 25% of all mice in this procedure): moderate discomfort throughout postnatal life. As mentioned above, these animals suffer motor-coordination problems and delayed growth, and will be used for experiments between postnatal day 8 (adolescence) - P30 (adulthood). In the rare cases they will not be used in experiments, they will be euthanized at P30 according to humane endpoints as outlined in Section J. ,

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

If the animals are not going to be used in other procedures first, they will be either maintained for stock breeding (only heterozygotes) or sacrificed for tissue harvesting. This entails harvesting brains from postnatal animals (adolescence – adulthood) for various measurement methods such as electrophysiology or stainings. Additionally, developmental aspects will be studied mainly in embryos of mid-late embryonal stage, which requires sacrifice of the pregnant mother. Harvested embryos will be used directly in experiments (e.g. stainings), or as a source of primary neurons for cell culture. Using *ex vivo* brain preparations for most of our experiments carries several advantages: Firstly, by slicing and staining for gene expression, one brain can be very efficiently used to investigate expression of several genes, with much more detail than would be possible otherwise (i.e. with *in vivo* imaging). In addition, this procedure keeps the discomfort to the animal to a minimum, as they do not suffer more than momentary additional discomfort during the euthanasia. Secondly, by using primary neurons derived from late-stage embryos for cell culture, we are able to use the brains very efficiently: One litter of embryos provides millions of primary neurons that can be cultured and studied in much greater detail and with much better environmental control than would otherwise be possible. Electrophysiology on acute brain slices of adult brains likewise allows for much better control over environmental variables (e.g. neurotransmitter levels) and measurements with single-cell precision that would not be

possible in *in vivo* recordings. Since the only additional discomfort the animal suffers from the euthanasia procedure is momentary and mild during the transport and anaesthesia, this is the optimal approach to investigate cellular and network properties while keeping animal discomfort to a minimum.

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

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

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.

Primary goal:
The primary goal of this research project is to unravel the function of language-related genes in the motor system of the mammalian brain. We aim to investigate the function of those genes on the molecular, cellular, physiological and behavioural level. In an advanced stadium of our research, we will attempt to systematically modify synapses or cell populations in living mice before the experiments, thus attempting to rescue the phenotype uncovered in earlier experiments. Thus, it is a prerequisite for the final experiments in the cellular, physiological and behavioural aspects of the projects.
General Design:
This appendix covers the experiments in which the mice get exposed to substances for targeted genetic modification (e.g. plasmids encoding GFP), labelling (e.g. anatomical tracers) or modification of protein function (e.g. interfering peptides). If the substances are broadly targeted, they will be administered by minimally invasive methods such as intraperitoneal injection. In cases where focal application in the brain is required, the animals will undergo a survival surgery procedure (small craniotomy + stereotactic injection) followed by a short period (< 3 weeks) to allow for recovery of the animal and transport/expression of the applied substance. In the special case that the modification makes neurons sensitive to stimuli such as light (when using virally-mediated optogenetics), a stimulator (e.g. glass fiber for light transduction) will be chronically implanted in the same survival surgery procedure.
Justification:
This approach is only going to be used in advanced stages of the project, meaning that it will be used to test the specific hypotheses built in previous, less invasive experiments (outlined in Appendix 1 & 2). For example, previous experiments have indicated that a specific synaptic pathway in a specific subpopulation of neurons is dysfunctional in Foxp2 mutants. In this case, we would try to rescue the phenotype by systemic intraperitoneal injection of a peptide known to block activity along this synaptic pathway. The animals would subsequently be sacrificed, and the function of these synaptic pathways would be studied using single-cell electrophysiology. If the electrophysiology shows a rescue of the phenotype, similarly treated animals would be tested in behavioural assays (Appendix 4). To summarise, this approach keeps the use of invasive techniques to a minimum, while being tightly controlled at each step. Where possible, globally-acting substances will be used because they can be administered via intraperitoneal injections or gavage, which considerably reduce discomfort for the animals. Only in cases where focal application to the brain is indispendible, we will use the survival surgery procedure. Also here, those experiments are strictly to verify previous theories or rescue the phenotype with targeted interventions. Together, these strict rules ensure that the use of invasive procedures for this project is kept to a minimum.

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

Animals will be taken from stock breeding (see Appendix 1 & 2). To achieve the best possible protection from contamination, the animals will be kept in a barrier environment such as individually ventilated cages (IVCs), fed sterilized chow food, and only handled under a flow hood. This setup is especially important for post-surgical care, because it greatly reduces the exposure of animals to potentially hazardous external influences.
All procedures will be carried out in the building complex of the central animal facility (Centraal Dierenlab, CDL) Nijmegen. The electrophysiology experiments necessitate transport of the live animal into the electrophysiology laboratory, which is located in the same building complex as the CDL.
Substance administration:
The mice will be given a dose of experimentally relevant substances, via one of the following routes:
a) Globally-acting substances (mild, one or several applications):
* Oral gavage: Mice will receive liquid substances, e.g. pharmaceutical compounds, via a blunt syringe.
* Addition to the food or drinking water for up to several weeks.
* Injection: One or several doses with globally targeted effects. Injection routes are either intraperitoneal, subcutaneous or intramuscular injections.
Justification a): These routes of administration are chosen to ensure that the animal receives the appropriate dose with minimal discomfort. If administration via the digestive tract is possible, the animal will receive the substance via food /drinking water (low dose) or oral gavage (high dose). If the substance cannot be absorbed via the digestive tract, it will be administered via injections at either intraperitoneal, subcutaneous or intramuscular injections, depending on e.g. the required dose and release properties. Together, these approaches ensure that globally acting substances are administered with as little discomfort as possible.
b) Brain-specific injection / implantation (moderate; single surgery with up to 3 weeks post-op survival time):
Animals are going to be anesthetized via inhalation of an isoflurane/carbogen (95% O_2, 5% CO_2) inhalation. Body temperature and depth of anaesthesia will be constantly monitored throughout the procedure. The head will be fixed in a stereotactical frame, followed by local anaesthesia and sterilization at the scalp, and the creation of a small craniotomy. Next, a small quantity of gene-expression vectors (viruses) or anatomical tracers will be delivered to stereotactically defined brain areas. Following injection / implantation, the wound will be re-sealed, disinfected, and local anaesthesia will be applied again. The animal will receive a general analgesic to aid recovery. Next, the animal will be kept warm until they show coordinated movement, then moved to their home cage and closely monitored for the next two hours. Antibiotics will be provided in the drinking water if needed. If the animal shows signs of post-operative distress, it will receive another dose of analgesics in 8-hour intervals and closely monitored. If the animal shows no betterment over the next 48 hours, it will be processed according to humane endpoints as outlined below. To allow for transport and expression of the stereotactically injected substances, a survival time of up to three weeks is necessary. At the end, animals will be either re-used in behavioural paradigms (see Appendix 4), or sacrificed for brain extraction and use in either stainings or electrophysiology.
Special case Optogenetics (Surgery plus stimulator implantation): If the injected virus changes the response qualities of the targeted neurons, as is the case in optogenetic modifications that make the neurons sensitive to light pulses, an appropriate stimulus delivery device (e.g. a glass fibre) will be implanted in the same procedure.
Justification b): This survival surgery procedure forms a crucial part of our project's advanced stage. Only when previous *ex vivo* experiments have provided a testable hypothesis about brain regions or neuronal subpopulations, we will proceed to test (and attempt rescue of the phenotype) those hypotheses with the *in vivo* experiments described here. Stereotactic injection under general anaesthesia allows to minimize animal discomfort while enhancing specificity, as it allows to precisely target specific brain regions through a minimal craniotomy (<1 mm^2). General anaesthesia, combined with extensive use of local anaesthesia and post-op analgesics, ensures that the animals suffer as little discomfort as possible during surgery and recovery. In addition, the strict post-operative surveillance regime we have planned will safeguard against undetected discomfort due to recovery.
Terminal anaesthesia, sacrifice and organ extraction, as described in Appendices 1+2 (non-recovery, <1 minute, once)
Embryo extraction: The pregnant mothers will be anesthetized with an anaesthetic such as pentobarbital, then quickly sacrificed. The embryos will be extracted to prepare *ex vivo* brain material for use in stainings, cell culture, or electrophysiological experiments. This procedure keeps discomfort

to the pregnant mother to a minimum, and the use of embryonal brains (especially for primary neuronal cultures) allows us to make very efficient use of the sacrificed animals, as millions of cells can be extracted and cultured for *in vivo* experiments. To add additional use per sacrificed animal, we will consider in our experimental plans to concurrently extract the pregnant mother's brain along with the embryos.
Brain extraction at adolescent-adult timepoints: The animal will be anesthetized via an anaesthetic such as pentobarbital, then quickly sacrificed. Subsequently, the brain will be quickly prepared out of the skull and immediately used for further experiments on the molecular and cellular levels. This approach ensures that the animals suffer as little discomfort as possible, while allowing us to measure gene expression in various parts of the brain's motor pathways. The use of *ex vivo* preparations allows for very precise localization and quantification of gene expression and circuit layout that would not be possible in any other way.
Special case electrophysiology: Transport to the lab (mild, 5 min, once), followed by terminal anaesthesia, decapitation and organ extraction (non-recovery, <1 minute, once): To fully understand the effects of language-related gene defects on the physiological level, i.e. the function of the brain's motor circuits, we aim to measure the electrophysiological properties of single cells and cell networks in acute brain slices of adolescent and adult mice. If done correctly, freshly extracted brain tissue can be cut into acute slices, in which most neurons keep living and functioning for up to 12 hours. During this time, the electrical activity of those neurons, and the networks they form, can then be probed in our specially equipped electrophysiology laboratory. The electrophysiological portion of the experiments necessitates transport of the live animal into our specially equipped electrophysiology laboratory, which is located in the same building complex as the CDL. The transport to our specially equipped electrophysiology lab is absolutely essential, since the technique requires immediate processing of the brain (< 1 minute after decapitation) in order to yield viable material. After processing, the tissue is extremely sensitive to movement, making any further transportation impractical. The animals will only experience mild discomfort during transport to the laboratory, which happens indoors and lasts for about 5 minutes. Once in the laboratory, they will be immediately anesthetized and decapitated, keeping discomfort to the animals to a minimum. This approach allows us to study the neurons in a well-controlled environment, and in much greater detail and with higher yield than would be possible *in vivo*.

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

The animals will be pre-selected to have the appropriate genotype and ideal age for the experiment, thus minimising the number of animals going into the procedure. The experiments described here will form the last step to mechanistically and directly prove influence of the targeted systems, and thus the hypotheses to test will already be closely focused by previous experiments. Because very specific hypotheses can be tested, we expect the animal numbers to be low.
In order to estimate the animal numbers required for experiments, we will use the program G*power (http://www.gpower.hhu.de/), assuming a clear and consistent change (effect size = 0.8), a power of 0.8 and a significance level of 0.05.

B. The animals

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

All experiments will be done in genetically modified mice. Mice will be taken from general breeding stock (described in Appendix 1, 1360 mice). If necessitated by the experiment, Foxp2-mutant mice from the heterozygote x heterozygote crossing that may suffer mild discomfort (bred under a

separate protocol, see Appendix 2, 1400 mice) will be used. Of those mice from Appendix 2, 600 (43% of 1400) are going to suffer moderate discomfort due to their genotype. This approach allows us to precisely control all parameters of the experiment before the substance administration. Since the mice used in this appendix are merely bred and maintained under Appendices 1 and 2, and are experimentally manipulated only as described here, their numbers will be registered under this Appendix (App. 3) and not for 1 or 2.
Furthermore, the mouse is an ideal model organism for this type of research, because it is a mammal and therefore phylogenetically closely related to humans, yet also uniquely amendable to genetic modifications. Mice share large genetic similarity with humans, and their brain's motor control circuitry has also been shown to be very similar. Taken together, these factors mean that mice are the model of choice for our project to completely characterize the influence of language-related gene defects from the molecular to the behavioural level.

Estimated numbers:

Since the described experiments are to test very focused hypotheses following from previous experiments described in the other appendices, we expect to need 2760 animals over the next five years , 21% of a total 13280 animals for the entire project. As stated above, we expect animal numbers to be low, because the experiments described herein will only be started once previous, less invasive experiments (such as *in vitro* and *ex vivo* preparations as described in Appendix 1 + 2) have resulted in clear, testable hypotheses. These experiments will enable us to make the crucial link between the cellular phenotypes found in the earlier experiments and the circuit-wise defects that result from them. Precise knowledge of circuit-level defects, in turn, will enable us to formulate mechanistic predictions about the specific behavioural aspects affected by mutations in language-related genes.

Life stages:

Animals will be used at the adolescent - adult stage (postnatal day > 14) for both gavage and surgery. We chose adolescent and adult timepoints because they provide optimal insight into the clinically relevant states of the mutant brain: Adolescence in mice roughly maps onto the first postnatal years in humans, which allows detailed research into the initial set-up of the motor system that is crucial for language in humans. Measuring in adulthood, on the other hand, is crucial to investigate the long-term effects of language-related gene defects.
To study embryonal development, in few cases, pregnant females will also be used for administration of neuroanatomical markers and pharmacological compounds. The former allows for "birthdating" neurons, which yields crucial insights into aspects of e.g. cortical development, where neurons with different functions are born sequentially. The latter is part of the rescue aspect of this procedure, as many defects observed in the adult brain have a developmental origin. Therefore, one promising prospect is to rescue these phenotypes by corrective action already during embryonal development.

Species	Origin	Maximum number of animals	Life stage
Mouse C57BL6J with genetic modifications in the Foxp2 gene (without discomfort)	Own breeding (see appendix 1,2)	600	Adolescence - adulthood (P14-P180)
Mouse C57BL6J with genetic modifications in the Foxp2 gene (with discomfort)	Own breeding (see appendix 2)	800	Adolescence - adulthood (P14-P180)
Mouse C57BL6J Mice with genetic modifications in other Foxp family member genes	Own breeding (see appendix 1)	800	Adolescence - adulthood (P14-P180)

Mouse C57BL6J with genetic modifications in genes whose human orthologues are relevant for neurodevelopmental disorders	Own breeding (see appendix 1)	400	Adolescence - early adulthood (P14-P30)
Mouse C57BL6J with genetic modifications to express transgenes such as LacZ, eGFP and Cre in a restricted manner	Own breeding (see appendix 1)	160	Adolescence - adulthood (P14-P180)

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:
- The substance administration experiments described in this procedure will only be started once previous experiments with cell cultures *in vitro* and sliced *ex vivo* preparations have resulted in a specific, testable hypothesis. Thus, we expect to require very low numbers of animals for the *in vivo* experiments described here.

Reduction:
- The animal strains will be carefully chosen with regards to the current needs of the experiments. For example, we reduce the variability in our

samples by measuring with littermate controls wherever possible. This allows us to reach stronger effect sizes with smaller groups of animals due to lower variability.
- The sophisticated post-surgery monitoring and care scheme planned here greatly enhances the animal's chances of survival, thus leading to a reduced number of animals required for the final experiment.
Refinement:
-To guarantee the best possible environment and minimize chances of contamination for the animals, they will be housed in sterilized individually ventilated cages (IVCs), receive sterilized food and drinking water, and only be handled under a flow hood. This housing regime is considerably more sophisticated than the filter top cages commonly used for similar experiments, and adds additional layers of isolation from potentially interfering outside influences, such as viral infections. This is especially relevant in the context of post-surgical care, as a better protection of the animals from outside influences enhances the post-surgical recovery prospects.
- The use of anaesthesia and analgesics during surgery and recovery keeps the animal's discomfort to a minimum. In addition, the strict monitoring scheme proposed for post-op care also serves to minimize animal discomfort during recovery.
- In addition, the use of anaesthesia in all terminal procedures actively minimizes animal discomfort. The use of the volatile anaesthetic isoflurane before electrophysiology experiments additionally ensures that the resulting brain slices, in which neurons live and function for several hours after preparation, are not influenced by anaesthetic residues.
- Rescue experiments are the ultimate proof that the gene of interest's function has been understood sufficiently to allow for reversal of the phenotype via targeted interventions. A successful rescue thus greatly enhances this research project's relevance for future clinical application.

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

1) Animals will be in a strictly isolated environment (IVCs, see above) to minimise chances of contamination. Animals will be monitored at least weekly, and any animal showing signs of excessive distress will be processed according to the humane endpoints outlined in section J.
During surgery, the animals will be kept under anaesthesia, sufficiently deep to feel no pain (periodically assessed via paw pinching reflex), but sufficiently light to still breathe autonomously. All surgery work will be carried out in aseptic conditions with sterilized tools, in specialized facilities of the CDL. All areas surrounding the craniotomy will be sterilized and locally anesthetized during and immediately after the surgery by application of a topical anaesthetic. At the end of the surgery procedure, the animal will receive a general analgesic to reduce pain during recovery. The analgesic (and/or antibiotics) will be re-administered if the animal shows any sign of distress in the weeks following surgery.

Repetition and Duplication

E. Repetition

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

Accommodation and care

F. Accommodation and care

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

[X] No

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

G. Location where the animals procedures are performed

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

[X] No > Continue with question H.

[] Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[] No > Continue with question I.

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

H. Pain and pain relief

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

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

I. Other aspects compromising the welfare of the animals

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

Post-administrative effects: In the case of general administration via oral gavage or injections, there are low risks of side-effects of pharmacological compounds. There is a very low risk of intra-abdominal damage and peritonitis from injections. Gavage will cause some minor discomfort on each administration. Global labelling agents such as BrdU (used for labelling dividing cells to "birthdate" neurons), might be carcinogenic; However, the time between administration and euthanasia (<2 weeks) is far too short for the animals to develop any tumors.
Post-surgical effects: There is a low risk of an inflammatory reaction at the craniotomy site, which will be closely monitored to ensure that the animal does not develop complications. Furthermore, animals will need to be housed alone in their home cage to prevent other animals from interfering with the sutures and healing of the surgery site, as they would during normal grooming.

Explain why these effects may emerge.

Post-administrative effects: Those mostly relate to needle trauma or mis-injection, both for oral gavage and injections. The risk of those effects is low, and will be reduced further by thorough training of any researchers and staff involved in the procedure.
Post-surgical effects: Since the surgery involves a craniotomy and, in some cases, implantation of a stimulus device onto the skull, the surgery site will have to heal during the recovery period. The risk of inflammation is low, and mostly relates to over-grooming of the sutures by the animal as part of normal grooming behaviour. The risks will be minimised by a sterile surgery environment, thorough training of any researcher and staff involved, and close monitoring of the animal post-surgery.

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

For oral gavage, injections, and surgery, thorough training by an experienced scientist and/or technician is crucial to minimising the risks during administration. Furthermore, the animals will be closely monitored during and after administration to ensure that they do not suffer undue distress.
Post-operative care: The animal will be kept warm (e.g. under a heating pad) following surgery until coordinated movement is visible, then placed in its home cage and monitored until alert. If the animal shows signs of pain or distress from the surgery site, it will be administered analgesics as needed. If any sign of inflammatory reaction is seen, the animal will be given antibiotics and anti-inflammatory medication as needed. Following the

surgery, the animals will be closely monitored at least daily and behavioural parameters (mood, activity, state of the surgical site) will be scored and noted.
In all cases, if the animal shows bodily (e.g. ruffled fur coat or wounds) or behavioural (e.g. limping, hunched back, or immobility) symptoms indicating undue distress, it will be euthanized according to the humane endpoints outlined below.

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.

General:
Despite the best countermeasures, animals might sometimes show signs of undue distress. Outward signs such as a ruffled fur coat or wounds, and behavioural signs such as limping, hunched back, or immobility will be taken as sign for undue distress, and the animal will be sedated and euthanized immediately.
Surgery:
For survival surgeries, the animal will be monitored daily post-surgery, and the state of the surgery site, behaviour, and level of activity will be scored and noted. If the animal does show any signs of distress, it will be given analgesics and antibiotics. In case the animal does not show betterment 48 hours after treatment, it will be euthanized.

Indicate the likely incidence.

The likely incidence is very low in the case of gavage side-effects such as needle trauma. It is low for adverse post-surgery outcomes such as inflammation.

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).

Substance administration: Animals are going to undergo one of the two following treatments: Administration (mild, <1 minute, once or several times 520 animals (19% of totally 2760 for this Appendix), of which 120 (4.3% of total 2760) with additional moderate discomfort due to their genotype. The administration via oral gavage and injection will cause a short moment of mild discomfort upon each administration. Gavage via drinking water will cause no additional discomfort.

Survival surgery / implantation (surgery: mild, ca. 2 h, recovery: moderate, ca. 1 week). 2240 animals (81% of totally 2760), of which 480 (17% of totally 2760) with additional moderate discomfort due to their genotype. The survival surgery and possible implantation will be done under anaesthesia, and pain will be reduced post-operation by application of analgesics as necessary. Yet, during the recovery period, the healing of the surgery site will induce moderate discomfort for the animal. Usually, the animals take a few days to recover, depending on the size of the craniotomy and the health status of the animal. After recovery, animals will generally not suffer further distress. Only in the case of chronic implantations there will be persistent mild discomfort due to constraints on the movement freedom.
Further use: Following substance administration, animals are either going to be euthanized in one of the two ways described below. This will be subject to a go/no-go decision (only if both behavioural and surgery procedures have produced encouraging data), and will be closely coordinated with the local animal welfare body.

In case of subsequent organ extraction: Transport, anesthesia and euthanasia as described in Appendix 1+2:
In case of electrophysiology: Transport to the lab (mild, 5 minutes, once). On the day of the experiment, animals will be placed in cardboard boxes with bedding material provided by the CDL staff. They will be quickly transported to our specially equipped electrophysiology lab in the Radboudumc, which is within the same building complex as the animal laboratory. The only discomfort the animals are going to experience during this time is movement and unfamiliar surroundings. Once in the laboratory, the animal will directly be euthanized and have their brains extracted as outlined below.
Euthanasia for tissue extraction (non-recovery, <1 minute, once): The animal will be sedated with an appropriate sedative such as isoflurane (in case of electrophysiology) or pentobarbital (for all other applications), and the absence of pain reception will be tested via e.g. forepaw pinching (absence of a reflex indicates total absence of pain sensation). Animals will be killed via cervical dislocation, decapitation, or transcardial perfusion, as the experiment necessitates. Typically, the brain will be extracted. In some cases, the brain will be extracted along with embryos of a defined developmental age.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

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

Animals will be used further in behavioural testing (see Appendix 4), or sacrificed an appropriate time after the substance administration for organ harvesting (typically brain and/or embryos). The latter entails harvesting brains from postnatal animals (adolescence – adulthood) for various measurement methods such as electrophysiology or stainings. Additionally, developmental aspects will be studied mainly in embryos of mid-late embryonal stage, which requires sacrifice of the pregnant mother. Harvested embryos will be used directly in experiments (e.g. stainings).

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

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

[X] Yes

Appendix
Description animal procedures

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

1 General information

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

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.

Primary goal:
The primary goal of this research project is to unravel the function of language-related genes in the motor system of the mammalian brain. We aim to investigate the function of those genes on the molecular, cellular, physiological and behavioural level. This animal procedure describes the behavioural experiments.
General Design:
This appendix covers the assessment of behavioural performance in the genetically modified mice described in the previous three appendices. To functionally validate the neuronal phenotypes of our mouse models *in vivo*, we plan to assess motor function, as well as general cognitive performance. This allows us to integrate the hypotheses generated from previous genetic and neuronal circuit-level research with data on behavioural output. Behavioural paradigms also allow experimental rescue, where a deficient phenotype is restored by targeted interventions, demonstrating the functional relevance of the hypothesis.
Our outcome parameters will be to precisely quantify a mutation’s influence on a specific task, matched to the type of circuitry that is thought to underlie the behaviour. For example, carriers of a global mutation in the Foxp2 gene are expected to have deficiencies in numerous parts of their motor circuitry. Therefore, experiments with these strains focus on challenging motor tasks, and targeted interventions for rescue experiments will manipulate specific parts of the motor circuitry.
In order to enrich the behavioural data with additional insight into the neurological underpinnings of the expected motor defects, the tested animal's brain is going to be extracted and assayed on micrometer scale after completion of the behavioral paradigm. As described in the other appendices, the animals will be terminally anesthetized, then quickly sacrificed. Subsequently, the brains will be extracted for quantification of single-cell level gene expression (i.e. via stainings). This approach allows us to link single-cell level gene expression to behavioural performance, providing unique insight into the mechanistic link between gene defect and behavioural phenotype.

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

Behavioural experiments (mild, several hours/day, <2 weeks):
Motor coordination will be assessed by using well-defined tasks: the accelerating rotarod and the tilted running wheel: These are non-stress motor-coordination paradigms.
Rotarod task: In this task the animal is placed on a rotating beam which rotates with increasing speed. Animals will be placed on this rotarod repeatedly, in several sessions per day, and scored for both duration (time to fall per day) and learning rate (increase in time to fall between days).

Rotarod experiments are only mildly discomforting and therefore can be used on consecutive days. We estimate to need approximately ten measuring days to properly assess motor performance.
Tilted running wheel: Tilted running wheel: This is a disc shaped running wheel which can be placed in the animals' home cages. As such running time (a measure of activity) and running bout length (a measure of exhaustion and coordination) can be assayed. The test is self-paced, meaning that the animal will freely engage in the running behaviour *ad libitum* and does not suffer any discomfort, not even handling or unfamiliar environments. However, the task requires that the animal be housed in its cage alone, which causes mild discomfort for the duration of the task
Justification Behaviour: These methods are among the least discomforting behavioural paradigms. Contrary to other behavioural paradigms such as fear conditioning, the animals will not be exposed to painful or otherwise aversive stimuli. Also, the assays mostly use naturally occurring behaviour patterns, therefore preventing potentially discomforting motivation paradigms such as water deprivation
Anaesthesia, euthanasia and organ extraction (non-recovery, >1 minute, once):
Following the end of the behavioural sessions, in some cases, the animal will be terminally anesthetized via an anaesthetic such as pentobarbital, then quickly sacrificed. Subsequently, the brain will be quickly prepared out of the skull and immediately used for further experiments on the molecular and cellular levels.
Justification brain extraction: This approach ensures that the animals suffer as little discomfort as possible, while allowing us to measure gene expression in various parts of the brain's motor pathways. The use of *ex vivo* preparations allows for very precise localization and quantification of gene expression and circuit layout that would not be possible in any other way. This approach furthers the goal of linking the behavioural performance to single-cell level properties such as gene expression, which can be assessed with stainings.

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

We expect the differences between animal groups to arise largely due to genotype. In order to estimate the animal numbers required for experiments, we will use the program G*power (http://www.gpower.hhu.de/), assuming a clear and consistent change (effect size = 0.8), a power of 0.8 and a significance level of 0.05.
We are, in some instances, able to use one animal for multiple behavioural tests, depending on the specific task. We will be able to minimize the number of animals both by proper choice of experimental paradigm and measurement method.

B. The animals

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

We will use transgenic mice bred and maintained at the CDL Nijmegen (described in Appendix 1 and 2). Of these, 720 are going to be derived from the breeding stock for the lines described in Appendix 1, and 720 are going to be taken from the breeding as described in Appendix 2. Of the Appendix-2-derived mice, 240 (33%) are going to suffer moderate discomfort due to their genotype. It should be noted that these animals are merely bred and maintained under Appendices 1 and 2, and the experimental manipulation of this group is going to happen as described in this Appendix (App. 4). Therefore, the animals of this group are going to be registered under Appendix 4, and not under Appendices 1 and 2.

Animals might have been subjected to procedures outlined in appendix 3 ("Substance Administration") prior to the behavioural assessments described here. The choice for this is highly dependent on experimental outcome and will lead to a go/nogo moment during the research. Nonetheless, we will conduct behavioral experiments with the animal numbers outlined here
Using mice bred in our own colony allows to select mice of optimal age for the experiment. Furthermore, the mouse is an ideal model organism for this type of research, because it is a mammal and therefore phylogenetically closely related to humans, yet also uniquely amendable to genetic modifications. Mice share large genetic similarity with humans, and their brain's motor control circuitry has also been shown to be very similar. Taken together, these factors mean that mice are the model of choice for our project to completely characterize the influence of language-related gene defects from the molecular to the behavioural level.

Estimated animal numbers and life stages:

We expect to require ca. 1440 animals between adolescent and adult ages (postnatal day 14 - 6 months of age) for this procedure, 11% of the total of 13280 mice for the entire project. These ages are ideally suited to probe the developmental trajectory of different modifications, allowing deep insight into the function of the motor circuitry of these mice. Contrasting adolescent and adult animals will allow us to identify developmental delays, a prominent feature in many human speech disorder phenotypes.

Species	Origin	Maximum number of animals	Life stage
Mouse C57BL6J with genetic modifications in the Foxp2 gene (without discomfort)	Own breeding (see appendices 1,2)	240	Adolescence - adulthood (P14-P180)
Mouse C57BL6J with genetic modifications in the Foxp2 gene (with discomfort)	Own breeding (see Appendix 2)	480	Adolescence - Early adulthood (P14-P30)
Mouse C57BL6J with genetic modifications in other Foxp family member genes	Own breeding (see appendix 1)	480	Adolescence - adulthood (P14-P180)
Mouse C57BL6J with genetic modifications in genes whose human orthologues are relevant for neurodevelopmental disorders	Own breeding (see appendix 1)	240	Adolescence - adulthood (P14-P180)

C. Re-use

Will the animals be re-used?

[X] No, continue with question D.

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

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

[] No

[] Yes > Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

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

- The mouse is at the moment the only mammal which can be used for stable genetic modifications of Foxp2. Therefore, we will need to use mice for our behavioural experiments. Mice are well suited for the type of experiments described here, are among the simplest organisms that can carry them out.
- The behavioural experiments described in this procedure will only be started once previous experiments with sliced *ex vivo* preparations have resulted in a specific, testable hypothesis. Thus, we expect to require very low numbers of animals for the *in vivo* experiments described here.

Reduction:

- The animal strains will be carefully chosen with regards to the current needs of the experiments. For example, we reduce the variability in our samples by measuring with littermate controls wherever possible. This allows us to reach stronger effect sizes with smaller groups of animals due to the lowered variability.
- Post-experiment euthanasia and brain extraction will allow to link gene expression to behavioural performance, further serving the goal of linking behavioural performance to properties of single cells in the brain's motor circuitry.

Refinement:

-To guarantee the best possible environment and minimize chances of contamination for the animals, they will be housed in sterilized individually ventilated cages (IVCs), receive sterilized food and drinking water, and only be handled under a flow hood. This housing regime is considerably more sophisticated than the filter top cages commonly used for similar experiments, and adds additional layers of isolation from potentially interfering outside influences, such as viral infections.
- Rotarod experiments have been conducted before on Foxp2 models, but only on Foxp2 mutants with a global deletion, lacking the protein in all tissues. We aim to investigate the effect of cell specific Foxp2 knockout animals (e.g. via Cre induction), or specific circuitry, e.g. Drd1+ cells in the striatum. Thus, our experiments will improve upon extant literature and probe much more specific functions of our genes of interest.
- Rescue experiments are the ultimate proof that the gene of interest's function has been understood sufficiently to allow for reversal of the phenotype via targeted interventions. A successful rescue thus greatly enhances this research project's relevance for future clinical application.

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

1) Animals will be kept in a strictly isolated environment (IVCs, see above) to minimise chances of contamination. They will be monitored at least weekly, and any animal showing signs of excessive distress will be euthanized according to the humane endpoints outlined in section J.
In the rotarod experiments, the rod will be placed only slightly higher than the mouse would jump voluntarily, minimising the possibility of distress due to falling. With object recognition or reaction tests, the chance for adverse effects is very low, as the animals will not be subjected to any painful or fear-inducing stimuli.

Repetition and Duplication

E. Repetition

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

Accommodation and care

F. Accommodation and care

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

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

G. Location where the animals procedures are performed

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

[X] No > Continue with question H.
[] Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

[X] No > Continue with question I.

[] Yes > Will anaesthesia, analgesiaor other pain relieving methods be used?

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

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

I. Other aspects compromising the welfare of the animals

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

Rotarod and cognitive function tests are considered as the mildest forms of behavioural assays. as the occurrence of accidental injuries is very low, we do not expect our animals to be adversely affected by these experiments.

Explain why these effects may emerge.

Despite the best countermeasures, some animals might develop illnesses, or experience social stress due to fighting among cagemates. There is a very low chance of accidental injuries from behavioural testing, for example falling from the rotarod.

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

The height of the rotarod is carefully chosen to just above the height after which mice would jump voluntarily. This greatly reduces the chances of accidental injuries due to the mouse falling off the rotarod.
If the animal shows bodily or behavioural symptoms indicating undue distress, it will be euthanized according to the humane endpoints outlined below.

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.

Despite the best countermeasures, animals might sometimes show signs of undue distress. Outward signs such as a ruffled fur coat or wounds, and behavioural signs such as limping, hunched back, or immobility will be taken as sign for undue distress.

Indicate the likely incidence.

The likely incidence is very low, since the mouse strains are overtly normal in health and behaviour, and are housed in specially isolated individually ventilated cages, reducing outside exposure. All behavioural paradigms are optimized for low risk to the animal's health, rendering the likelihood of injuries during the behavioural paradigms very low.

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).

Behavioural assays (mild, several occurrences over consecutive days): The animals will be handled, i.e. picked up, placed on the rotarod / behavioural assay location, then observed for the course of a session. Subsequently, the animal will be placed in its home cage and brought back to the housing room. The assays will be repeated on consecutive days, at approximately the same time of day to insure minimal influence of circadian rhythm.
Euthanasia for tissue harvesting (non-recovery): The animal will be sedated with an appropriate sedative such as pentobarbital or isoflurane, and the absence of pain reception will be tested via e.g. forepaw pinching (absence of a reflex indicates total absence of pain sensation). Animals will be killed via cervical dislocation, decapitation, or transcardial perfusion, as the experiment necessitates. Subsequently, the brain will be isolated, fixated and used for further post-mortem measurements (e.g. stainings).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

[] No > Continue with Section 3: 'Signatures'.

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

After the end of behavioural assays, the animals might be euthanized to harvest their brains, e.g. for stainings, allowing to correlate neuronal phenotypes to behavioural data. This requires a terminal procedure for the animals.

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

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

[X] Yes

Appendix 3: Substance administration

Category	Experiment	Mouse Lines	# Experi-ments	Groups	Animals/ group	Subtotal	Total	Appen-dix total
Mice with genetic modifications in the Foxp2 gene (with discomfort, bred under Appendix 2)	Oral gavage	2	2	3	10	120		
	Viral injections	2	2	3	20	240		
	Tracer injections	2	2	3	20	240	**600**	
Mice with genetic modifications in the Foxp2 gene (without discomfort, bred under Appendix 2)	Oral gavage	4	2	2	10	160		
	Viral injections	4	2	2	20	320		
	Tracer injections	4	2	2	20	320	**800**	
Mice with genetic modifications in other Foxp family member genes (bred under Appendix 1)	Oral gavage	4	2	2	10	160		
	Viral injections	4	2	2	20	320		
	Tracer injections	4	2	2	20	320	**800**	
Mice with genetic modifications in genes whose human orthologues are relevant for neurodevelopmental disorders (bred under Appendix 1)	Oral gavage	2	2	2	10	80		
	Viral injections	2	2	2	20	160		
	Tracer injections	2	2	2	20	160	**400**	
Mice with genetic modifications to express transgenes such as LacZ, eGFP and Cre in a restricted manner (bred under Appendix 1)	Virus injection	2	2	2	20	160	**160**	***2760***

Appendix 4: Behaviour

Category	Experiment	Mouse Lines	# Experi-ments	Groups	Animals/ group	Subtotal	Total	Appen-dix total	
Mice with genetic modifications in the Foxp2 gene (with discomfort, bred under Appendix 2)	Motor function assay	2	2	2	30	240	**240**		
Mice with genetic modifications in the Foxp2 gene (without discomfort, bred under Appendix 2)	Motor function assay	4	2	2	30	480	**480**		
Mice with genetic modifications in other Foxp family member genes (bred under Appendix 1)	Motor function assay	4	2	2	30	480	**480**		
Mice with genetic modifications in genes whose human orthologues are relevant for neurodevelopmental disorders (bred under Appendix 1)	Motor function assay	2	2	2	30	240	**240**	**1440**	**13280**

* = Embryo extractions are calculated differently, because they mainly serve as source for primary neuronal cultures. We calculate over time rather than in block-experiments: 1 pregnant mother / week, 50 weeks / year. Thus, 100 animals indicate a steady supply of 1 litter a week for 2 years. For each experiment, only one mother of one genotype is sacrificed.

† = Counts towards the total of Appendix 3.

‡ = Counts towards the total of Appendix 4.

2015-2016	2016-2017	2017-2018	2018-2019	2019-2020
Breeding	Breeding	Breeding	Breeding	Breeding
Behavior (Foxp2)	Behavior (Foxp2)	Behavior (others)	Behavior (others)	Behavior (others)
		01-2018, last moment start new experimental procedures		07-2020 – end of project license

Appendix 1: Breeding without discomfort

Category	Experiment	Mouse Lines	# Experi-ments	Groups	Animals/ group	Subtotal	Total	Appen-dix total	Grand Total
Mice with genetic modifications in the Foxp2 gene	Embryo extraction *	5				500			
	Electrophysiology	5	5	2	20	1000			
	Postnatal extraction	5	2	2	20	400	1900		
Mice with genetic modifications in other Foxp family member genes	Embryo extraction *	5				500			
	Electrophysiology	5	5	2	20	1000			
	Postnatal extraction	5	2	2	20	400	1900		
	To Appendix 3 †						(800†)		
	To Appendix 4 ‡						(480‡)		
Mice with genetic modifications in genes whose human orthologues are relevant for neurodevelopmental disorders	Embryo extraction *	4				400			
	Electrophysiology	4	5	2	20	800			
	Postnatal extraction	4	2	2	20	320	1520		
	To Appendix 3 †						(400†)		
	To Appendix 4 ‡						(240‡)		
Mice with genetic modifications to express transgenes such as LacZ, eGFP and Cre in a restricted manner	Embryo extraction *	2				200			
	Postnatal extraction	2	4	2	10	160	360		
	To Appendix 3 †						(160†)	5680	

Appendix 2: Breeding with discomfort

Category	Experiment	Mouse Lines	# Experi-ments	Groups	Animals/ group	Subtotal	Total	Appen-dix total
Mice with homozygous Foxp2-mutation (with discomfort)	Electrophysiology	4	5	1	20	400		
	Postnatal extraction	4	5	1	20	400	800	
	To Appendix 3 †						(600†)	
	To Appendix 4 ‡						(240‡)	
Mice with heterozygous Foxp2 mutation or wildtype (without discomfort)	Embryo extraction *	4				1000		
	Electrophysiology	4	5	2	20	800		
	Postnatal extraction	4	5	2	20	800	2600	
	To Appendix 3 †						(800†)	
	To Appendix 4 ‡						(480‡)	3400

11.

Year 1	Year 2	Year 3
Breeding mutant animals	Breeding mutant and possible transgenic animals	Breeding mutant and possible transgenic animals
Cellular localization – developmental profile	Behavioral experiments on mutant and transgenic animals Rescue experiments	
Electrophysiological characterization of mutant animals, primary neuronal cultures	Electrophysiological characterization of transgenic animals (cell / region specific mutation)	Assess viability of rescue on cellular, electrophysiological and behavioural level.

DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer 2015-0038
2. Titel van het project: :Language related genes in neurodevelopment and brain function.
3. Titel van de NTS: Onderzoek naar de invloed van taalgenen op de hersenontwikkeling en hersenfunctie.
4. Type aanvraag:
   nieuwe aanvraag projectvergunning
5. Contactgegevens DEC:
   - Naam DEC: RUDEC
   - Telefoonnummer contactpersoon: [redacted] bereikbaar op maandag, dinsdag, en donderdag van 9:00 tot 15:00 uur
   - Mailadres contactpersoon: [redacted]
6. Adviestraject:
   ontvangen door DEC: 23-04-2015
   in vergadering besproken: 04-05-2015
   vragen gesteld: 11-05-2015
   antwoorden en aangepaste aanvraag ontvangen op 21-05-2015 en herbesproken in DEC-vergadering op 02-06-2015
   aanvraag compleet: 02-06-2015
   anderszins behandeld: aangepaste aanvraag en finaal advies zijn op 22 juli 2015 in een schriftelijke e-mailronde voorgelegd aan de DEC-leden voor instemming.
   besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: n.v.t.
   aanpassing aanvraag: 21-05-2015
   advies aan CCD: 06-08-2015
7. Eventueel horen van aanvrager: n.v.t.
   - Datum
   - Plaats
   - Aantal aanwezige DEC-leden
   - Aanwezige (namens) aanvrager
   - Strekking van de vraag / vragen
   - Strekking van het (de) antwoord(en)
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag
8. Correspondentie met de aanvrager
   - Datum: 11-05-2015
   Strekking van de vragen:

Niet-technische samenvatting:

3.1. In het projectvoorstel leggen de onderzoekers uit dat foxp2 een transcriptiefactor is die betrokken is bij veel processen in de hersenen, en dat mutaties hierin leiden tot ernstige spraak- en taalstoornissen. In de niet-technische

samenvatting wordt het gepresenteerd als taalgen. De commissie vindt dit geen juiste weergave, en verzoekt de onderzoekers dit preciezer te formuleren. Hierdoor zal het voor leken ook begrijpelijker zijn waarom taalstoornissen in een niet-pratend dier onderzocht kunnen worden.
3.3 Het aantal muizen komt niet overeen met de aantallen uit de beschreven dierproeven. De onderzoekers worden verzocht dit in overeenstemming met elkaar te brengen. Zie ook de vraag hieronder over onderdeel B van dierproef 1.
3.5 De onderzoekers worden verzocht de percentages muizen te noemen bij de differentiatie naar mate van ongerief en er zorg voor te dragen dat deze overeenstemmen met die genoemd onder 3.4.

Project Proposal:

De commissie adviseert u geen namen van personen te vermelden.
3.1. De onderzoekers willen in de komende jaren meer genen gaan onderzoeken, en noemen Foxp1, Tbr1 en Cntnap2. Kunnen zij beter onderbouwen waarom deze genen onderzocht zullen worden? Bevinden zij zich in dezelfde pathway als foxp2? Leiden mutaties in deze genen ook tot motorische problemen?

Description of Animal Procedures:

- Dierproef 1, onderdeel B. De aantallen in de tabel en de tekst komen niet overeen. In de tekst is sprake van 5680 dieren, terwijl in de tabel 5410 dieren staan. De onderzoekers vermelden hier dat er 2960 dieren voor dierproef 4 gebruikt zullen worden, terwijl er in dierproef 4 2880 dieren gebruikt zullen worden afkomstig uit dierproef 1 en 2. De onderzoekers worden verzocht deze aantallen in overeenstemming met elkaar te brengen.

Worden in dierproef 3 en 4 alleen dieren gebruikt die al geteld zijn in dierproef 1 en 2? Betekent dit dat in het hele project 5680 (of 5410 zie hierboven?) + 3400 = 9080 (of 8810?) dieren worden gebruikt voor dierproeven? De onderzoekers worden verzocht het totaal aantal dieren duidelijker te vermelden en in overeenstemming te brengen met het in de niet-technische samenvatting genoemde aantal.
- Dierproef 3, onderdeel K. De onderzoekers maken onderscheid naar mate van ongerief, maar vermelden hierbij niet de percentages dieren die het betreft. Zij worden verzocht dit toe te voegen.
- Dierproef 4, onderdeel A eerste vraag. Waarom kijken de onderzoekers naar cognitieve functie? Dit volgt niet logisch uit de doelstelling van dit experiment. Indien de onderzoekers het gebruik van testen voor cognitieve functie kunnen onderbouwen, worden zij verzocht hun keuze voor de genoemde parameters te onderbouwen in relatie tot de functie van de onderzochte genen.
- Dierproef 4, onderdeel A tweede vraag. Welke andere motor coördinatie taak overwegen de onderzoekers te gebruiken, en met welk ongerief zal dit gepaard gaan?

- **Datum antwoord: 21-05-2015**

  Non-technical summary:

  Section 3.1: You requested clarifications of the link between Foxp2 and language disorders. We have amended the section with a more detailed description of the relation between Foxp2 and language disorders. We also included a more detailed explanation of the link to animal research.

Section 3.2: You remarked that the numbers were not consistent between NTS, project proposal and animal procedures. We have re-calculated and corrected the numbers in all parts of the project. For clarity, we added an overview table to the project proposal.
Section 3.5: You requested addition of the animal number percentages. We have amended the text to include percentages, grouped by discomfort. We have amended Section 3.4 to match the percentages.

Project proposal:

You pointed out that the project proposal should not include names. We have amended the text throughout to replace names with equal formulations. We also changed the citation style from (Author, Year published) to (Title, Journal, Year Published). Along with the short summaries that accompany all cited publications, this citation style should help clarify the meaning of the citation.
Section 3.1: You requested clarifications to the rationale of studying additional genes such as Foxp1, Tbr1 and Cntnap2, along with Foxp2. We have amended the text to include a short summary of the current knowledge about each gene, and their links to speech and language disorders.
In order to further clarify the animal numbers, we have added an overview table to Section 3.4.2 (Outline of the animal procedures). This table gives an overview of animal strains, experimental lines, expected group size, and under which animal procedures animals are going to be registered.

Description of animal procedures:

Appendix 1, Section B: You pointed out that the animal numbers between text and tables do not match. We have re-calculated the animal numbers throughout the project and amended the text and tables to match. We also added a detailed description of the planned movements of animals between different animal procedures.
Appendix 3, Section B: You requested that the descriptions also include the percentage of animals experiencing discomfort. We have amended the text to include the percentage of animals suffering discomfort. Likewise, we amended the texts of the other animal procedures to include which percentage (if any) of the animals suffers discomfort due to their genotype.
Appendix 4, Section A, first question: You requested clarifications about the link between experimental question and the planned experiments for cognitive function. Since this part is not yet crucial to the project as a whole, we have decided to remove it from the project proposal. We have amended the text to focus on the motor learning aspects. We included the reduction in requested animals for this procedure in our re-calculation of the animal numbers, and amended the animal table to reflect the change.
Appendix 4, Section A, second question: You requested clarification about the nature of the second motor learning experiment, and the level of discomfort it includes. We have amended the text to clarify that a tilted running-wheel paradigm will be used. This paradigm will cause discomfort similar to the previously named rotarod task, i.e. mild discomfort, over a comparable timeframe.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

9. Eventuele adviezen door experts (niet lid van de DEC): n.v.t.

- Aard expertise

- Deskundigheid expert
- Datum verzoek
- Strekking van het verzoek
- Datum expert advies
- Expert advies

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig.
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.

C. Beoordeling (inhoud):

1. Het project is:

    - uit wetenschappelijk oogpunt verantwoord

2. De in de aanvraag aangekruiste doelcategorie is in overeenstemming met de hoofddoelstelling.
3. De DEC onderschrijft het belang van het voorgenomen onderzoek, dat er op gericht is om de effecten van mutaties in taal-gerelateerde genen op moleculair, cellulair, neurofysiologisch en gedragsniveau te identificeren. Het onderzoek zal resulteren in kennis over de rol van met name het Foxp2 gen en een aantal andere genen waarmee het interacteert in de zich ontwikkelende hersenen van de muis. Voorts wordt duidelijk op welke manier defecten in deze genen resulteren in gedragsafwijkingen. De bevindingen in dit onderzoek kunnen meer algemeen gebruikt worden in andere onderzoekslijnen waarin getracht wordt het mechanisme te ontrafelen waardoor genmutaties leiden tot gedragsafwijkingen. Mutaties in Foxp2 en de genen waarmee het interacteert leiden bij mensen tot spraakstoornissen. Een spraakstoornis heeft een grote impact op het sociaal functioneren. Maatschappelijk is dit onderzoek van belang, omdat de resultaten op termijn mogelijk kunnen leiden tot betere diagnose en therapieën voor mensen die lijden aan een spraakstoornis met een genetische oorzaak, wat resulteert in gezondheidswinst en kostenreductie door het terugdringen van sociaal isolement. De realisatie van de verwachte resultaten vertegenwoordigt in de ogen van de DEC een substantieel belang.
4. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project. Het moleculaire onderzoek zal leiden tot het identificeren van doelen voor celresearch en electrofysiologie. Gedragsexperimenten zijn afhankelijk van de identificatie van cellulaire mechanismen die door de mutatie zijn aangetast. Deze aanpak leidt tot natuurlijke go/no go momenten. De DEC acht de geschetste tijdslijn geloofwaardig en haalbaar. De samenwerking tussen onderzoeksgroepen binnen de Nijmeegse Max Planck en Donders Instituten zal kunnen leiden tot een integraal beeld van het effect van mutaties in de onderzochte genen. De gekozen aanpak leidt dan ook tot betrouwbare uitspraken over het effect van mutaties in foxp2 en de genen waarmee het interacteert op hersenen en gedrag van muizen.
5. Er is geen sprake van bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren.
6. Het ongerief als gevolg van de dierproeven is, op grond van eigen ervaring, door de aanvragers realistisch ingeschat en geclassificeerd. Het matige ongerief wordt voornamelijk veroorzaakt

door bijkomen uit de anesthesie en herstel na operatie in de verschillende muizenstammen en fenotypen en door motor-coordinatieproblemen en groeiachterstand in de homozygote Foxp2-muizen. Dit ongerief wordt in beide gevallen als matig ingeschat en treft 27% van de gevraagde 13.280 muizen. De overige dieren ondervinden licht ongerief ten gevolge van bijvoorbeeld gedragstesten en handelingen als doden onder anesthesie door cervicale dislocatie, decapitatie, of transcardiale perfusie. Het cumulatief ongerief voor de muizen in de beschreven dierproeven is dus juist ingeschat.

7. Er zijn geen methoden die de voorgestelde dierproeven geheel of gedeeltelijk zouden kunnen **vervangen.** De doelstelling van het project kan niet gerealiseerd worden zonder proefdieren of door gebruik van minder complexe diersoorten. Voor de primaire neuronale celkweken zijn embryo's nodig. Hersenontwikkeling, interacties tussen hersencellen en het functioneren van hersencircuits kunnen alleen ex vivo of in vivo in hersenen worden onderzocht, waarvoor dieren nodig zijn waarvan de hersenen voldoende op mensen lijken. Gedragsafwijkingen kunnen alleen in dieren worden onderzocht met een toereikend gedragsrepertoire. Het is hier van belang te vermelden dat ex vivo en in vivo experimenten gedeeltelijk vooraf worden gegaan door experimenten in kweken van geïmmortaliseerde cellen (dus in vitro onderzoek) op basis waarvan hypotheses worden geformuleerd voor verder ex vivo en in vivo onderzoek.
8. In het project wordt optimaal tegemoet gekomen aan de vereiste van de **vermindering** van dierproeven. Het maximale aantal te gebruiken dieren is realistisch ingeschat en proportioneel ten opzichte van de gekozen strategie en de looptijd. Resultaten uit celkweekexperimenten zullen gebruikt worden om gerichte hypothesen te onderzoeken in de diermodellen op het gebied van elektrofysiologie en gedrag. De sequentie moleculair > cellulair > electrofysiologie > gedrag biedt voldoende mogelijkheden om nut en noodzaak van de volgende stap te overwegen. De DEC is het eens met het beschreven onderzoeksmodel en de onderbouwing van het aantal benodigde dieren. De DEC is van oordeel dat de verwoorde doelstellingen kunnen worden behaald met maximaal 13280 muizen in 5 jaar.
9. Het project is in overeenstemming met de vereiste van de **verfijning** van dierproeven. De experimentele handelingen bij de dieren zullen worden uitgevoerd door hierin getrainde onderzoekers, waardoor de stress voor de dieren zoveel mogelijk wordt beperkt. Driekwart van de dieren ondergaat licht ongerief. De DEC is ervan overtuigd dat de dierproeven zo humaan mogelijk worden uitgevoerd.
   Er is geen sprake van belangwekkende milieueffecten.
10. De niet-technische samenvatting is een evenwichtige weergave van het project, zelfstandig leesbaar, beknopt en begrijpelijk geformuleerd.

D. Ethische afweging

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

Met dit fundamentele onderzoek kunnen belangrijke nieuwe inzichten worden verkregen in de specifieke effecten van (mutaties in) genen welke betrokken zijn in de ontwikkeling van spraak en taal (bij mensen) op onderscheiden niveau's in de zich ontwikkelende hersenen van muizen. Opheldering van de wijze waarop Foxp2 en gerelateerde genen functioneren in de muis, zal een basis kunnen leggen voor het begrip hoe defecten in deze genen resulteren in gedragsafwijkingen en spraak- en taal-stoornissen in de mens. Dergelijke resultaten zijn essentieel om gerichte

methodieken voor diagnose en therapie te kunnen ontwerpen. Het is dan ook aannemelijk dat de resultaten van het voorgenomen onderzoek in belangrijke mate kunnen bijdragen aan de uiteindelijke ontwikkeling van verbeterde modaliteiten voor diagnose en therapie bij mensen die lijden aan een spraakstoornis met een genetische oorzaak. De mogelijke ultieme gezondheidswinst bij patiënten met dergelijke aandoeningen vertegenwoordigt volgens de commissie een substantieel belang. Het beschreven onderzoek kan niet zonder proefdieren worden verricht.

Tegenover dit substantiële belang staat het gegeven dat 27% van de maximaal 13.280 muizen matig ongerief zal ondervinden, ten gevolgen van fenotype en/of bijkomen uit narcose en herstel na een operatieve ingreep. De overige dieren ondervinden licht ongerief. De commissie is er van overtuigd dat bij de dierproeven adequaat invulling zal worden gegeven aan de vereisten op het gebied van de vervanging, vermindering en/of verfijning van dierproeven. Het resterende ongerief is onvermijdelijk, wil men de doelstellingen kunnen realiseren. De experimenten komen qua design overeen met wat in het onderzoeksveld gebruikelijk is.

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

E. Advies

1. Advies aan de CCD
   - ☐ De DEC adviseert de vergunning te verlenen

2. Het uitgebrachte advies is gebaseerd op consensus.