	Dossier: AVD11500202519049	
		Aanwezig
1	NTS	X
2	Aanvraagformulier	X
3	Projectvoorstel	X
4	Bijlage beschrijving dierproeven	4X
5	DEC-advies	X
6	Ontvangstbevestiging	X
	Evt. Vragen CCD aan aanvrager	3X
	Evt. antwoorden aanvrager	2X
7	Beschikking en vergunning	X

Een instantie van UMC Utrecht Universiteit Utrecht

NIET-TECHNISCHE PROJECTSAMENVATTING

Naam van het project	Bestuderen welke rol onze eigen afweer heeft in het ontwikkelen van (chronische) pijn
NTS-identificatiecode	NTS-NL-849966 v.1, 07-01-2026
Land	Nederland
Taal	nl
Duur van het project, uitgedrukt in maanden.	60
Trefwoorden	Pijn Zenuwstelsel Afweersysteem
Doel(en) van het project	Fundamenteel onderzoek: Zenuwstelsel Omzettinggericht en toegepast onderzoek: Zenuwziekten en psychische aandoeningen van de mens

DOELSTELLINGEN EN VERWACHTE VOORDELEN VAN HET PROJECT

Beschrijf de doelstellingen van het project (bijvoorbeeld het aanpakken van bepaalde wetenschappelijke onduidelijkheden, of wetenschappelijke of klinische behoeften).

Chronische (ofwel aanhoudende) pijn komt wereldwijd voor bij ongeveer één op de vijf volwassenen. Chronische pijn kan het gevolg zijn van problemen in het afweersysteem, door veroudering of door een bepaalde ziekte, zoals reuma en artrose. Chronische pijn wordt ook vaak veroorzaakt door zenuwbeschadigingen, bijvoorbeeld bij kanker of diabetes of als bijwerking van een kankertherapie. Ook bij dieren is chronische pijn een groot probleem. Bijvoorbeeld 25 tot 80% van de honden krijgt artrose en chronische pijn door artrose is één van de hoofdoorzaken van kreupelheid bij paarden. Op dit moment bestaan er geen goede medicijnen waarmee we chronische pijn kunnen behandelen. Het is van groot belang om beter te begrijpen hoe chronische pijn ontstaat, zodat we op basis van deze kennis nieuwe medicijnen kunnen ontwikkelen.

Pijn ontstaat als gevolg van een ingewikkelde wisselwerking tussen het zenuwstelsel en het afweersysteem. Afgelopen jaren hebben we verschillende mechanismen ontdekt die belangrijk zijn om deze wisselwerking optimaal te laten verlopen, zodat de kans op chronische pijn minder is of chronische pijn zelfs behandeld kan worden. De belangrijkste bevindingen van de afgelopen jaren hebben ons geleerd dat als de communicatie van zenuwcellen en afweercellen niet goed verloopt, dat er chronische pijn kan ontstaan. In dit project willen we beter begrijpen hoe samenwerking tussen zenuwen en het afweersysteem helpt om pijn te stoppen, wat er misgaat bij langdurige pijn, en laten zien dat we deze samenwerking kunnen beïnvloeden om pijn te verlichten. Dit willen we onderzoeken door te:

1) Bepalen welke veranderingen er plaats vinden in immuun en zenuwcellen tijdens het verdwijnen van pijn en chronische pijn.
2) Hoe we de interacties tussen deze twee celtypes kunnen beïnvloeden om nieuwe behandelingen voor chronische pijn te ontdekken

Omdat er diverse soorten chronische pijn bestaan, zullen wij verschillende ziektes met chronisch pijn nabootsen in muizen en ratten. Zo willen we beter begrijpen hoe deze chronische pijn ontstaat en of de therapeutische werking van nieuwe stoffen de pijn en ontsteking kan remmen bij verschillende ziektes of alleen in een bepaalde aandoening (bv alleen artrose).

Welke potentiële voordelen kan dit project opleveren? Leg uit hoe de wetenschap vooruit kan worden geholpen of mensen, dieren of het milieu uiteindelijk voordeel kunnen hebben bij het project. Maak, waar van toepassing, een onderscheid tussen voordelen op korte termijn (binnen de looptijd van

Er is een groot tekort aan effectieve en veilige behandelingen voor patiënten met chronische pijn. Mochten we een nieuwe behandeling vinden die klachten van chronische pijn kan verlichten, of zelfs de oorzaak van chronische pijn kan omkeren, dan is het grootste voordeel te behalen bij de patiënten. De kwaliteit van leven zal verbeteren en ook zijn er economische voordelen voor de maatschappij, aangezien er minder mensen zullen uitvallen (qua werk) door klachten als gevolg van de pijn. Artsen zullen dus ook voordeel hebben bij ons onderzoek als we beter chronische pijn begrijpen en kunnen behandelen.

Daarnaast zullen de farmaceutische/biotech-bedrijven interesse hebben in ons werk. Momenteel werken we al met een paar bedrijven samen om te onderzoeken of we nieuwe geneesmiddelen

het project) en voordelen op lange termijn (die mogelijk pas worden bereikt nadat het project is afgerond).	kunnen ontwikkelen om chronische pijn te behandelen. Wij verwachten ook voordelen voor de wetenschap op de korte termijn. Aangezien de wisselwerking tussen neuronen en ons afweersysteem ook belangrijk is in verschillende hersenziektes (bijvoorbeeld Parkinson), kunnen andere onderzoekers ook interesse hebben in ons werk. Ook de proefdieren zelf en werknemers in dierenlaboratoria kunnen baat hebben bij ons onderzoek. Momenteel is het lastig te zien of een proefdier pijn heeft en of dit goed behandeld wordt. Ons onderzoek kan hierbij helpen op lange termijn.

VOORSPELDE SCHADE

In welke procedures worden de dieren gewoonlijk gebruikt (bijvoorbeeld injecties, chirurgische procedures)? Vermeld het aantal en de duur van deze procedures.

Een aantal dieren zal worden gedood voor het verzamelen van weefsels/cellen voor gebruik in celkweken en microscopie of om terug te injecteren in muizen.

Het grootste gedeelte van de dieren wordt gebruikt in een pijnmodel (door middel van een injectie of operatie). Dit kunnen modellen zijn voor zenuwschade, botpijn of pijn gerelateerd aan een ontsteking of een infectie door een ziekteverwekker. Om pijnmechanismes te onderzoeken, kunnen de dieren injecties krijgen op een lokale plek, zoals het ruggenmerg (intrathecaal) om daar direct de neuronen/afweercellen te beinvloeden of meer systemisch door een stof in de bloedbaan te spuiten (intraveneus). Tijdens de proef zullen de dieren verschillende gedrags- en of pijnmetingen ondergaan.

Aan het eind van de proef zullen de dieren gedood worden.

Wat zijn de verwachte gevolgen/nadelige effecten voor de dieren, bijvoorbeeld pijn, gewichtsverlies, inactiviteit/verminderde mobiliteit, stress, abnormaal gedrag, en wat is de duur van die effecten?

Het grootste gedeelte van de dieren in onze experimenten zal pijn ervaren. Dit is onvermijdelijk, omdat we onderzoek doen naar het oplossen van chronische pijn. Waar mogelijk, bijvoorbeeld na een operatie, geven we de dieren pijnstilling.

De dieren krijgen een injectie om botpijn, ontstekingspijn of zenuwpijn te induceren. Deze pijn is in de meeste gevallen kortdurend. Maar we onderzoeken ook wat er gebeurt als de pijn chronisch word. Als de pijn langdurig aanhoudt zal dit matige pijn zijn. Behalve als het experiment langer duurt dan 4 weken, zullen we de langdurige matige pijn inschalen als ernstige pijn.

Daarnaast zullen de dieren behandelingen krijgen om te onderzoeken of we de pijn kunnen behandelen. Dit kan d.m.v. een injectie in een bloedvat, in een ontstoken gewricht of d.m.v. een ruggenprik

Welke soorten en aantallen dieren zullen naar verwachting worden gebruikt? Wat zijn de verwachte ernstgraden en de aantallen dieren in elke ernstcategorie (per soort)?

Soort:	*Totaal aantal*	*Geraamde aantallen naar ernstgraad*			
		Terminaal	*Licht*	*Matig*	*Ernstig*
Muizen (Mus musculus)	5230	126	2001	2388	715
Ratten (Rattus norvegicus)	606	100	122	0	384

Wat gebeurt er met de dieren die aan het einde van de procedure in leven worden gehouden?

Soort:	*Geraamd aantal te hergebruiken, in het habitat-/houderijsysteem terug te plaatsen of voor adoptie vrij te geven dieren*		
	Hergebruikt	*Teruggeplaatst*	*Geadopteerd*
Muizen (Mus musculus)	350	0	0
Ratten (Rattus norvegicus)	130	0	0

Geef de redenen voor het geplande lot van de dieren na de procedure.

Als wij aan het eind van een experiment geen weefsels hoeven te verzamelen voor verder onderzoek in het lab, willen wij de dieren wanneer mogelijk zo veel mogelijk gebruiken voor het oefenen van technieken of voor het verzamelen van cellen voor celkweek. We verwachten 100 muizen en 100 ratten nodig te hebben gedurende 5 jaar, voor het leren/oefenen van technieken. Hiervoor zullen we zoveel mogelijk gebruik maken van surplus dieren. We zullen dieren ook aanbieden voor hergebruik, zodat andere onderzoekers ze kunnen gebruiken voor deze doeleinden. Gebaseerd op afgelopen jaren, verwachten we 5% van onze dieren die hiervoor in aanmerking komen (~250 muizen, ~30 ratten). Dus in totaal komen we dan op ~350 muizen en ~130 ratten voor hergebruik.

TOEPASSING VAN DE DRIE V'S

1. Vervanging
Beschrijf welke diervrije alternatieven op dit gebied voorhanden zijn en waarom zij niet voor het project kunnen worden gebruikt.

onderzoeksvragen over de rol van een bepaald gen of eiwit in specifieke celtypes beantwoorden in het lab. Deze antwoorden helpen ons om de keuzes te maken voor de proeven die nog uitgevoerd moeten gaan worden in muizen of ratten.

Ook is ons lab nu onderdeel van een project waarbij we alternatieven voor dierproeven aan het testen zijn, om "pain-in-a-dish" te onderzoeken. Mede hiervoor zijn we een model aan het opzetten waarin we van stamcellen van mensen, zenuwcellen kunnen maken. Wanneer dit model goed loopt, kunnen wij potentiële nieuwe behandelingen eerst testen op deze cellen waardoor alleen de meest veelbelovende behandelingen in dieren getest hoeven te worden en er minder dieren nodig zijn.

De in vitro-testen worden gedaan op cellijnen, organoïden, maar ook in cellen waarvoor dieren gedood zijn. Dit is nodig omdat relevante eigenschappen van bepaalde celtypes soms verloren gaan in het doorgroeien van cellen, waardoor verse materialen nodig zijn.

De in vitro-proeven zijn belangrijk, maar ze zijn ook beperkt omdat je maar naar één of twee celtypes kunt kijken. Hierdoor kun je niet onderzoeken wat de invloed van een celtype op de omgeving of zelfs het hele lichaam is. Chronische pijn is het resultaat van een ingewikkelde wisselwerking tussen onder andere het immuunsysteem en het zenuwstelsel. Momenteel is het nog niet mogelijk dit in het lab zonder dieren na te bootsen.

2. Vermindering
Leg uit hoe de aantallen dieren voor dit project zijn bepaald. Beschrijf de stappen die zijn genomen om het aantal te gebruiken dieren te verminderen en de beginselen die zijn gebruikt bij het opzetten van de studies. Beschrijf, waar van toepassing, de praktijken die gedurende het hele project zullen worden toegepast om het aantal dieren die in overeenstemming met de wetenschappelijke doelstellingen werden gebruikt, tot een minimum te beperken. Deze praktijken kunnen bijvoorbeeld bestaan uit proefprojecten, computermodellen, het delen van weefsel en hergebruik.

Voor de fok van de muizen maken we gebruik van de meest optimale fokstrategieën om voor zover mogelijk alleen de dieren te fokken die we nodig hebben voor onze experimenten.

Met behulp van statistiek bepalen we wat de beste groepsgrootte is in deze studies, zodat we met de kleinste hoeveelheid proefdieren de onderzoeksvraag kunnen beantwoorden.
Verder verminderen we het aantal dieren dat we nodig hebben door niet voor elke proef altijd alle controledieren mee te nemen. In veel experimenten zijn de controlegroepen hetzelfde, dus door gebruik te maken van de kennis die we al hebben kunnen we het aantal dieren dat in nieuwe experimenten nodig is verminderen.

Ook zullen we op meerdere manieren kijken naar mechanismes die een rol spelen bij pijn. We kunnen bijvoorbeeld na het einde van een proef in de verzamelde weefsels van een dier naar de rol van meerdere celtypes kijken en zo mogelijk meerdere vragen beantwoorden.

Verder maken we zoveel mogelijk gebruik van mannelijke en vrouwelijke dieren. Maar wanneer er grote verschillen zijn, is het mogelijk dat we maar naar één geslacht kijken om het aantal dieren dat nodig is te verminderen.

Wanneer we aan het eind van een proef geen weefsel hoeven te verzamelen voor verder onderzoek in het lab, kijken we of de dieren gebruikt kunnen worden voor het aanleren van nieuwe technieken of als bron voor celkweek. Dit kan binnen onze eigen onderzoeksgroep, maar de dieren kunnen ook worden aangeboden aan anderen voor hergebruik.

3. Verfijning
Geef voorbeelden van de specifieke maatregelen (bv. verscherpte monitoring, postoperatieve behandeling, pijnbestrijding, training van dieren) die in verband met de procedures moeten worden genomen om de welzijnskosten (schade) voor de dieren tot een minimum te beperken. Beschrijf de mechanismen om gedurende de looptijd van het project

Wij ontwerpen onze experimenten altijd zo, dat de proeven zo kort mogelijk zijn. Dit doen we omdat in het algemeen het ongemak voor de dieren samenhangt met de leeftijd en de duur van de proef.

Verder maken we zoveel mogelijk gebruik van automatische monitoring in een thuiskooi. D.m.v. een camera in de deksel van de kooi kan er continue bekeken worden hoe het met de muis gaat, bijvoorbeeld of er geen problemen zijn met lopen. In eerder onderzoek deden wij (en andere onderzoekers) dit door een muis in een aparte omgeving te zetten, om vervolgens te onderzoeken of lopen niet is aangedaan. De monitoring in thuiskooi heeft als voordeel dat de dieren minder stress ervaren van het overbrengen naar een nieuwe omgeving.

Als een experiment het toelaat maken wij ook gebruik van pijnstilling, bijvoorbeeld om pijn na een operatie te verminderen. Ook controleren wij de dieren extra na een operatie.
Plastic onderdak en tissues worden in de kooi geplaatst ter verrijking.

nieuwe verfijningstechnieken in gebruik te nemen.	Het welzijn van de dieren wordt minimaal twee keer per week gecontroleerd. Ook maken wij zoveel mogelijk gebruik van de minimale effectieve dosering van behandelingen, dit doen we om bijwerkingen zoveel mogelijk te verkleinen. Om individuele huisvesting zoveel mogelijk te voorkomen, plaatsen we meerdere dieren per kooi, zodat ze minder stress ervaren. En wanneer mogelijk maken we gebruik van een 'maatje' mocht een dier toch alleen overblijven.
Licht de keuze van de soorten en de bijbehorende levensstadia toe	Wij zullen voor het grootste deel van onze experimenten gebruik maken van een veel gebruikte muizenstam (C56Bl/6). In deze muizen kun je chronische pijn condities nabootsen zoals die ontstaan na zenuwschade of ontsteking in de mens. Hierdoor kunnen we beter onderzoeken welke mechanisme belangrijk zijn voor het ontwikkelen van chronische pijn. Muizen, en deze stam, worden het meest gebruikt voor pijnstudies, waardoor we al veel weten over deze modellen. Daarnaast maakt het gebruik van muizen mogelijk om een bepaalde aanpassing te doen, door bijvoorbeeld een stukje DNA (een gen) erin of eruit te halen, om zo te onderzoeken of dat gen/eiwit belangrijk is voor het ontwikkelen van chronische pijn. Het kan voorkomen dat we soms muizen van een andere stam gebruiken. Dit doen we dan omdat een bepaalde erfelijke aanpassing alleen bestaat in die bepaalde stam, en we daarmee voorkomen veel extra muizen (en tijd) te moeten gebruiken om die aanpassing te krijgen in een C56Bl/6 achtergrond. De meeste muizen die we gebruiken, zijn 7 tot 26 weken oud. Voor een aantal experimenten (bijvoorbeeld zenuwpijn bij diabetes) kunnen de experimenten maximaal tien maanden duren. We maken voor een aantal experimenten gebruik van ratten. Dit doen we omdat bepaalde (operatie)technieken niet mogelijk zijn bij muizen omdat deze te klein zijn.

VOOR EEN BEOORDELING ACHTERAF GESELECTEERD PROJECT

Project geselecteerd voor BA?	ja
Termijn voor BA	31-01-2031

Reden voor de beoordeling achteraf

Bevat ernstige procedures	ja
Maakt gebruik van niet-menselijke primaten	
Andere reden	
Toelichting van de andere reden voor de beoordeling achteraf	

AANVULLENDE VELDEN

Link naar de eerdere versie van de NTS buiten het EC-systeem	

Centrale Commissie Dierproeven

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

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

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*	11500
☐ Nee > *U kunt geen aanvraag doen*	

1.2 Wat voor aanvraag doet u?

☒ Nieuwe aanvraag > Ga verder met vraag 1.3

☐ Wijziging > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.1

☐ Melding > Vul hiernaast het AVD nummer van uw vergunde project in en ga verder met vraag 2.2

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

Naam instelling of organisatie	Universiteit Utrecht			
Titel, voorletters en achternaam van de portefeuillehouder	Titel [redacted]	Voorletters [redacted]	Achternaam [redacted]	☒ Dhr ☐ Mw
E-mailadres contactpersoon	info@ivd-utrecht.nl			
Titel, voorletters en achternaam van diens gemachtigde (indien van toepassing)	Titel n.v.t.	Voorletters	Achternaam	☐ Dhr ☐ Mw
E-mailadres gemachtigde				

Vul de gegevens van het postadres in.

Straat- en huisnummer	Instantie voor Dierenwelzijn Utrecht		50
Postcode en plaats	3584CJ	UTRECHT	
Postbus, postcode en plaats	80.125	3508TC	UTRECHT

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

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

1.5 *(Indien van toepassing)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	(Titel) Naam en voorletters	[redacted]	☐ Dhr ☒ Mw
	Functie	[redacted]	
	Afdeling		
	Telefoonnummer	[redacted]	
	E-mailadres	[redacted]	
1.6 *(Indien van toepassing)* Vul hier de gegevens in van de persoon aan wie de portefeuillehouder de verantwoordelijkheid inzake de algemene uitvoering van het project en de overeenstemming daarvan met de projectvergunning heeft gedelegeerd.	(Titel) Naam en voorletters		☐ Dhr ☐ Mw
	Functie		
	Afdeling		
	Telefoonnummer		
	E-mailadres		
1.7 *(Optioneel)* Vul hier de gegevens in van de Instantie voor Dierenwelzijn	Telefoonnummer	030-2531569	
	E-mailadres	info@ivd-utrecht.nl	
1.8 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 Gaat uw aanvraag over een *wijziging* op een vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn?	☒ Nee > Ga verder met vraag 3
	☐ Ja > Geef hieronder kort de wijziging en de onderbouwing daarvan weer. Geef in de originele formulieren (niet-technische samenvatting, projectvoorstel en bijlage dierproeven) duidelijk aan (bij voorbeeld in een andere kleur) waar de projectaanvraag wijzigt. Ga daarna verder met vraag 6.
2.2 Gaat uw aanvraag over een *melding* op een vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn?	☐ Nee > Ga verder met vraag 3
	☐ Ja > Geef hieronder weer wat deze melding inhoudt en ga verder met vraag 6.

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?	Startdatum	01-11-25
	Einddatum (t/m)	5 jaar na verlenen vergunning
3.2 Wat is de titel van het project?	Deciphering neuro-immune interactions and metabolism in pain regulation	
3.3 Wat is de titel van de niet-technische samenvatting?	Ontcijferen hoe de stofwisseling van cellen van het zenuwstelsel en immuunsysteem de interactie tussen deze cellen beïnvloed en daarmee chronische pijn.	
3.4 Wat is de naam van de Dierexperimentencommissie (DEC) van voorkeur?	Naam DEC	DEC Utrecht
	Postadres	Postbus 85500, 3508 GA UTRECHT
	E-mailadres	dec-utrecht@umcutrecht.nl

4 Factuurgegevens

4.1 (Indien factuuradres afwijkt van de gegevens uit vraag 1.3) Vul de gegevens van het factuuradres in.

Naam:	UU-ASC		Afdeling:
Straat:			Huisnummer:
Postcode:		Plaats:	
Postbus:	80.011	Postcode: 3508TA	Plaats: UTRECHT
E-mail:	asc.factuur@uu.nl		

4.2 (Optioneel.) Vul hier het ordernummer van de instelling in.

Ordernummer:
CB.841910.3.01.011

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel	Aantal bijlage(n) dierproeven 3
☒ Niet-technische samenvatting	

Overige bijlagen, indien van toepassing

☐ Melding machtiging

☐

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD en per post naar de Centrale Commissie Dierproeven (voor adresgegevens zie website)

Ondertekening door de portefeuillehouder namens de instellingsvergunninghouder of gemachtigde (zie 1.8). 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 C van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

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

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for 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 the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMCU, Utrecht University
1.3	Provide the title of the project.	Deciphering neuro-immune interactions in pain regulation

2 Categories

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

- ☒ Basic research
- ☒ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or animal
- ☐ 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 2.1.

Chronic pain affects roughly 20% of the global population (~3 million in the Netherlands) and arises from inflammation (e.g. rheumatoid arthritis, inflammatory bowel disease; **inflammatory pain**), nerve injury (surgery, diabetes, cancer, chemotherapy; **neuropathic pain**) and bone disorders (osteoarthritis, cancer bone metastases; **bone pain**) (Yong et al., 2022; Gobina et al., 2019). Existing drugs (opioids, NSAIDs) provide meaningful relief to only ~30% of patients and often cause intolerable side effects (Geurts et al., 2017). Chronic pain is also highly prevalent in animals (e.g., arthritis in dogs and horses), underscoring translational relevance (Anderson et al., 2018;

Frisbie et al., 2006; Neogi et al., 2017). A major barrier to better therapies is limited understanding of why pain sometimes resolves, but in many cases becomes, independent of the initiating insult, persistent (Denk et al., 2014; Kavelaars & Heijnen, 2021)

Clinically diverse causes often produce similar, persistent pain, implying convergent mechanisms that maintain neuronal hypersensitivity after the initial trigger has resolved (Denk et al., 2014). For example, ~30% of rheumatoid arthritis patients remain painful after inflammation subsides, and 10–40% of osteoarthritis patients report persistent pain after total knee replacement (Wylde et al., 2011; Taylor et al., 2010). Thus, chronic pain can become independent of the original stimulus (Kavelaars & Heijnen, 2021). Evidence across these diverse clinical chronic pain conditions point to maladaptive changes in sensory neurons. Yet chronic pain is not only a neurological problem, evidence indicate a strong contribution of the immune system, where cells and mediators interacts with neurons at multiple anatomical levels of the sensory nervous system to sustain neuronal hypersensitivity (Price et al., 2019; Reichling et al., 2019). Importantly, data suggest that pain resolution is not merely passive; it is an active process requiring coordinated immune–neural signaling. Our prior CCD work (2021–2025; AVD11500202010805) and related studies identified key neuro-immune elements that promote resolution or drive chronification.

Neuro-immune mechanisms at three sites

Neuro-immune interactions that shape pain occur at three key sites: **the periphery** (skin, joint, nerve endings/axons), **the dorsal root ganglia** (DRG; sensory neuron cell bodies), and the **central nervous system** (spinal cord and brain) (see Fig. 1). This neuro-immune signaling at each site can differentially drive resolution or chronification of pain. Our prior CCD work (2021–2025; AVD11500202010805), and related studies, identified key neuro-immune elements at the periphery and DRG/spinal cord that promote resolution or contribute to chronification:

In the periphery, immune triggers (sites of injury/inflammation) at peripheral nerve endings or along axons, can initiate maladaptive neuron activation. Several immune-derived factors actively trigger sensory neurons and initiate cascades that lead to chronic pain. For example, immune cells like macrophages and neutrophils infiltrate the tissue and secrete inflammatory mediators (e.g bradykinin, histamine, ATP, neurotrophins, cytokines) that can activate and sensitize sensory neurons (Lizarraga et al., 2025; Kanashiro, 2020). Moreover, complement activation and autoantibodies drive pai[redacted]ic pain conditions. Elevated complement in OA synovial fluid correlates with pai[redacted]utoantibodies (IgG/IgM) induce pain through activation of complement (e.g., C2 [redacted] uo, 2024), and peptides produced extensively in type 2 diabetes mellitus (such as human IAPP) cause chronic pain (Albariqi, 2023). Passive transfer studies implicate pathogenic IgG in fibromyalgia, neuropathic pain and post-viral syndromes, likely via complement and direct neuronal/immune effects (Goebel et al., 2021; Lacagnina et al., 2024; Chen et al., 2024; Moen et al., 2025). Conversely, anti-inflammatory mediators (e.g., IL-10, resolvins) suppress neuronal activity and promote resolution of inflammatory and neuropathic pain. Immune cells (macrophages, T cells) are phenotypically plastic and can switch between pro- and anti-inflammatory states, thereby promoting, maintaining, or resolving pain. How these state changes fail in chronic pain and whether they are therapeutically targetable remains to be determined.

Dorsal root ganglia (DRG) and spinal cord: Peripheral triggers also induce inflammatory states within the DRG and spinal cord. Pro-inflammatory macrophages accumulate in the DRG in OA and neuropathic models an[redacted] [redacted]t pain, whereas tissue-healin[redacted]siently in the DRG during resolutio[redacted] [redacted] et al., 2020; Lim et al., 202[redacted] We discovered that immune cells, [redacted] macrophages, can actively resolve inflammatory pain. Macrophage-to-neuron mitochondrial transfer and CD200R–iSec1 receptor–ligand signaling are essenti[redacted] CD200R, iSec1, or macrophages prevents pain resolution despite cessation of inflammatio[redacted]ther inhibitory immune receptors (e.g., LAIR1) and T cells also appear relevant to regulat[redacted]ow how. Moreover, aggregating peptides (e.g., human IAPP) and neuronal mitochondrial dysregulation promote chronic pain. hIAPP induces [redacted] nerve damage and mechanical hypersensitivity and IAPP aggregates can activate complemen[redacted] Moreover, neuronal mitochondrial dysfunction (e.g., increased ATPSc[redacted] [redacted] immune infiltration in the DRG and impairs pain resolution[redacted] [redacted] hese data indicate that disturbances in sensory neurons cause [redacted] c pain

Collectively, these findings support the **central hypothesis that coordinated neuro-immune communication actively mediates pain resolution and that disruption of these interactions drives chronic pain.** Remaining gaps include how peripheral immune triggers (e.g., complement, autoantibodies, aggregates) interact with DRG neuro-immune circuits and inhibitory receptor pathways to determine whether pain resolves or becomes chronic. For example, specific immune triggers in the periphery (e.g. complement) may induce failure in pain resolution mechanisms comprising of neuro-immune interactions in the DRG. Additionally, changes in sensory neurons and immune cells, for example inhibitory receptor/ligand pairs may promote or limit proper neuro-immune communication, affecting pain regulation.

To address these knowledge gaps, we will use mouse models of transient (resolving) pain and chronic pain and also utilize patient-derived biological materials (if available) to identify factors associated with either resolved or persistent pain. Candidate factors are tested in transient pain models to determine whether a certain factor contributes to successful pain resolution. For example, does removal of the identified factor (e.g. the inhibitory receptor LAIR) cause transient pain to transition into chronic pain? In addition, chronic pain models allow us to investigate what goes wrong when pain becomes chronic and whether targeting a specific pain-inducing factor can broadly attenuate chronic pain (e.g. activating inhibitory receptors, such as LAIR). By using different chronic pain models we will also test whether a role in pain regulation is limited to particular pain conditions such as inflammatory, neuropathic, or bone pain. This integrative approach enables us to dissect the context-specific roles of neuro-immune interactions and identify promising therapeutic targets for diverse chronic pain syndromes.

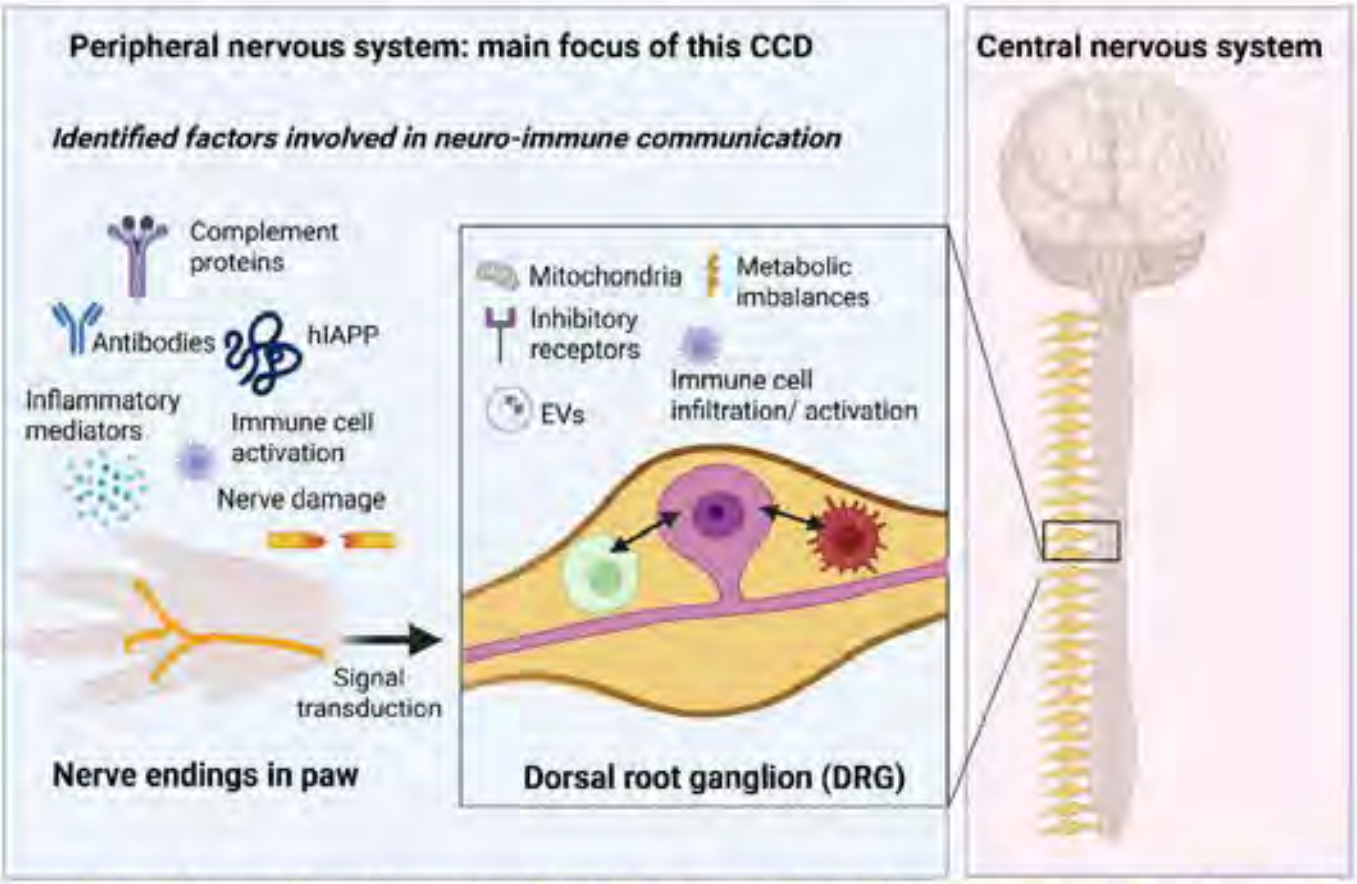

Figure 1. Main findings that we have achieved the last 5 years in the peripheral nervous system. We have identified several factors that can induce pain at the level of the nerve endings, but also found factors important for pain regulation at the level of the dorsal root ganglion to regulate neuro-immune communication.

3.2 Purpose

3.2.1 Describe the project's immediate and ultimate goals. Describe to which extent achieving the project's immediate goal will contribute to achieving the ultimate goal.

- If applicable, describe all subobjectives

Central goal: To elucidate how specific neuro–immune interactions actively mediate pain resolution and how failure of those interactions causes chronic pain, and to provide proof-of-concept that these interactions are targetable to restore resolution and reverse chronic pain.

Specific intermediate goals:

1. Dissect neuro–immune interactions that drive resolution versus chronification of pain.

We will define which immune cell types, receptors/ligands and secreted mediators at the periphery and in the DRG/spinal cord determine successful pain resolution versus persistent pain across inflammatory, neuropathic and bone-pain models. We will do this by using loss- and gain-of-function manipulations (genetic deletion/overexpression, antibody blockade/activation, cell depletion/repletion) to determine causal roles of each interaction in enabling resolution or driving chronification.

Key interactions to be tested include:

- Macrophage and T cell interactions with sensory neurons
- Inhibitory receptor–ligand pairs on immune cells and neurons
- Complement activation downstream of IgG/IgM and IAPP aggregates;
- Neuron-intrinsic mitochondrial regulators that modulate immune recruitment/phenotype.

2. Proof-of-concept of targetability of defined neuro–immune mechanisms in chronic pain.

We will select top candidate interactions from goal 1 (e.g., restoring macrophage mitochondrial transfer via CD200R–iSec1, activating inhibitory receptors such as LAIR1/CD200R, blocking complement activation, neutralizing pathogenic IgG or IAPP, or normalizing neuronal mitochondrial function) and test whether we can reinstate resolution mechanisms and reverse established chronic pain in mouse models. We expect to test max 1 to 2 candidates/per year.

Interventions will be mechanistically interrogated (cellular phenotypes, mitochondrial function, inflammatory mediators, and behavioral readouts) to confirm target engagement and pathway restoration.
Emphasis is proof-of-concept: demonstrate that modulating these neuro-immune interactions is sufficient to restore resolution or reverse chronic pain, without moving into drug development or clinical translation within this project.

3.2.2 Provide a justification for the project's feasibility.

To reach our goals, we will use cell-based (in vitro) models, human patient-derived biomaterials, knowledge from literature and animal models to better understand how neurons-immune communication regulate pain. We will study different types of pain, since each type of pain has both shared and unique neuron-immune communication. For practical reasons, and based on our own and others' research, we focus on three main types of pain that are common in humans and each shows both shared and distinct neuro-immune mechanisms (more extensively explained in appendix 1):

1. **Inflammatory pain** (pain caused by inflammation),
2. **Bone pain** (pain caused by osteoarthritis or bone cancer), and
3. **Neuropathic pain** (pain caused by diabetes, chemotherapy or tissue trauma).

Model selection follows a predefined decision tree to avoid unnecessary or inefficient animal use (see figure 3 and [redacted] established that monocytes /macrophages resolve inflammatory pain we will test their contribution to bone pain or neuropathic pain.

Experiments are staged and conditional: predefined go/no-go criteria (Fig. 3) permit early termination of non-promising experiments, minimizing animal numbers.

In addition, we **need transient and chronic pain models** (see appendix 1 and 2). To understand the outcome of communication between immune cells and sensory neurons, both transient and chronic models are included so we can determine causal roles in the transition to chronic pain. This is supported by our previous work that demonstrated that immune cells have opposing effects on pain depending on the model (see background).

The objectives that we described are achievable in an efficient fashion and without causing unnecessary discomfort to the animals, because we use widely validated, well-characterized rodent models and standardized behavioral and molecular endpoints that our team routinely employs (detailed in methods and supported by prior publications).

We already run complementary non-animal and human studies that directly feed candidate selection and go/no-go decisions for the CCD animal work. Ongoing efforts include clinical studies in patients with chronic pain to identify resolution-associated and chronification-associated targets, human iPSC-derived sensory-neuron assays to test target effects on neuronal sensitization, and additional non-animal in vitro screens. These activities are grant-supported (including long-term Growth Fund support for the iPSC sensory-neuron program for up to 10 years), and their results are used to prioritize, and where appropriate, halt or support in vivo lines of work before animal use.

We have collaborations in neuroscience, immunology and the clinic within the UMCU, UU, and abroad that secure important expertise, knowledge and input from the clinics tha[redacted]ents proposed here.
Finally, we are part of an innovative European pain consortiu[redacted]at helps us to achieve our objectives efficiently and give access to importa[redacted]D students from our own group have visited some of the lab[redacted] learn new animal models (e.g. CIBP) or alternatives for ani[redacted].

Our current team consists of 2 PostDocs, 5 PhD students, 2 animal technicians all dedicated to investigate neuro-immune communication in pain regulation. This demonstrates that we have enough people to perform the proposed experiments. Grants to these investigators include funding for animal work. All colleagues work closely together, to try to collect multiple tissues (paw, DRG, spinal cord) and cell types from a single animal thereby addressing multiple research questions while reducing the overall number of animals used. We expect other people to join our team in the next five years to further support our goals. In addition, all experiments are planned and executed in a step-by-step fashion. Each experiment will be discussed biweekly or weekly in our research team (and with collaborators) to determine the next logical step to address our questions as efficiently as possible. Based on these experiments we may also decide not to continue investigating one specific route when the experiments show for instance, that a gene, a cytokine, or an immune cell does not appear to be central in chronic pain development or does not show significant potential as therapeutic intervention (see figure 3 for go/no-go). By taking this approach, we are confident that within 5-years we can give answers to our research questions and that we have found new avenues for intervening in neuro-immune pathways to treat or prevent chronic pain.

3.2.3 Are, for conducting this project, other laws and regulations applicable that may affect the welfare of the animals and/or the feasibility of the project?

☒No

☐Yes > Describe which laws and regulations apply en describe the effects on the welfare of the animals and the feasibility of the project.

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3.3 Relevance

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

Chronic pain remains one of the most under-recognized and under-treated medical problems of the 21st century. It is estimated that 20% of the adult world population suffers from chronic pain and that this condition severely debilitates patients (Eur. J. Pain, 2006, 10, 287-333 ; National Center for Health Statistics Hyattsville. Health, United States. With Chartbook on Trends in the Health of Americans. 68-71. 2006. Hyattsville, MD). Besides the large

clinical implications of chronic pain, there is also a large economic burden. The estimated total annual costs of pain amount to $560 to $635 billion annually, which includes health care costs and loss of productivity associated with pain symptoms and this will most likely increase due to the aging population. The prevalence of chronic pain, and the associated costs are greater than those costs associated with cancer, type-2 diabetes mellitus (T2DM) and heart disease together (Gereau et al. 2011). Moreover, current pain treatments commonly fail to improve the quality of life and causes severe side effects. The standard line of treatment includes opioids, with its use (e.g. oxycodon) increased 4-fold since 2010 in The Netherlands (Weesie et al., 2020). Opioids often result in addiction and worsening of symptoms long-term (Simonin et al., 2016). Therefore, new pain therapies are needed. To reach this goal, better understanding of the mechanisms underlying chronic pain is needed. This knowledge will drive the development of novel treatments that not only reduce pain in a more targeted manner, but target the underlying causes of pain, enabling more effective and durable treatments rather than merely providing temporary symptom relief. To develop more effective, lasting therapies for different types of chronic pain, it is essential to thoroughly understand the dynamic interactions and metabolic processes of sensory neurons and immune cells during both pain resolution and pain chronification. For this, we will use *in vitro* models (e.g neuro-immune cultures), human patient materials (e.g. blood, cerebrospinal fluid, human patient skin for e.g. immunostaining including nerve innervation), and induced pluripotent stem cells (iPSCs), but these systems do not fully capture the complexity of the pain system. Therefore, animals play an indispensable role in achieving our scientific goals. The justification to focus on inflammatory-, neuropathic- and bone-pain is that these conditions are clinically relevant. In addition heterogeneity of clinical pain: Inflammatory, neuropathic and bone pain have distinct initiating causes and clinical courses. Using multiple models ensures our findings are relevant across these clinically diverse conditions rather than restricted to a single etiology. Although different insults can converge on common pathways, they also engage distinct neuro-immune triggers (e.g., complement and autoantibodies in OA, metabolic aggregates in diabetic neuropathy). Comparing models lets us separate convergent, generalizable mechanisms of resolution from etiology specific pathways. Demonstrating that a neuro-immune interaction (e.g., macrophage mitochondrial transfer, inhibitory receptor signaling, complement activation) is necessary and/or sufficient across multiple models strengthens causal inference and supports that the mechanism is broadly relevant to chronic pain.

Pain is a complex, system-wide process involving intricately connected neuronal and immune networks that cannot be fully recapitulated in vitro. Animal models allow us to:

-Investigate pain resolution and pain chronification in the context of an intact nervous and immune system, capturing dynamic, cell-to-cell and tissue-to-tissue interactions.
-Experimentally manipulate genetics, immune cell subsets, and metabolic pathways in vivo to determine causal relationships.
-Translate findings into proof-of-concept therapeutic strategies prior to clinical application, ensuring both efficacy and safety.

The latter point and importance of our objectives is highlighted by previous work in our group. Based on insights we gained in our animal models of pain regulation, we have developed synerkines, a fusion protein of two inflammatory cytokines. These proteins showed promising results in our chronic pain models and on the basis of this work a phase 1/2 trial is planned in patients with complex regional pain syndrome in 2026 to test the efficacy and safety of IL4-10 Synerkine (this research is not part of this CCD, but is investigated under AVD11500202519050). In addition, we have now started some collaborations with pharmaceutical companies to drive therapy development based on the insights we gained with our animal models. As an example, based on the combination of our patient-material derived research and mouse models of chronic pain, we have now identified the complement system as an immune-driver of pain in OA. This has led to a collaborative effort with Argenx, with an antibody targeting Complement component C2 to treat OA pain, currently being further developed. Overall, developing therapies that target the root causes of chronic pain will address a critically unmet medical need, improving quality of life for patients and reducing the economic burden of persistent pain conditions. The basic insights and proof-of-concepts gained from our approach will inform the development of targeted, durable pain therapies, moving the field beyond symptomatic management to disease-modifying strategies.

3.3.2 Who are the project's stakeholders? Describe their specific interests.

Firstly, the wellbeing of **experimental animals** is very important to us, as they are directly affected by the research. Therefore, we will always consider the three Rs: Replacement, Reduction and Refinement (more detail in Appendices).

Patients, as the ultimate beneficiaries, have a vested interest in research that leads to better pain management strategies and improved quality of life. Understanding pain mechanisms through animal models provides critical insights that could lead to more effective therapies for those suffering from chronic pain conditions.

Clinicians are particularly invested in how the findings can translate into improved patient care, especially in pain management. They rely on preclinical studies, including those using animal models, to better understand pain mechanisms and evaluate potential treatments that could be applied in clinical settings.

Industry stakeholders, including pharmaceutical and biotech companies, are interested in the development and commercialization of new pain therapies. Depending on our research, we may collaborate with industry stakeholders. They may support the research through funding, collaboration, or by integrating findings into their drug development pipelines. Their focus is on identifying promising targets and interventions that can be brought to market to address unmet needs in pain treatment. Some examples of ongoing collaborations to develop new drugs for various chronic pain conditions, based on our research outcomes, are Argenx and Ono Pharmaceutical.

Other (pain) **researchers** can benefit from our findings by integrating these in their own research plans and refining protocols/techniques to enhance both data quality and animal welfare. Moreover, our findings are relevant for researchers in the field of neuroscience and immunology in general and specifically for the field studying neurodegenerative diseases including, but not limited to, multiple sclerosis, Alzheimer's disease and Parkinson's disease, all diseases in which metabolic status of neurons/immune cells are worsening the disease outcome.

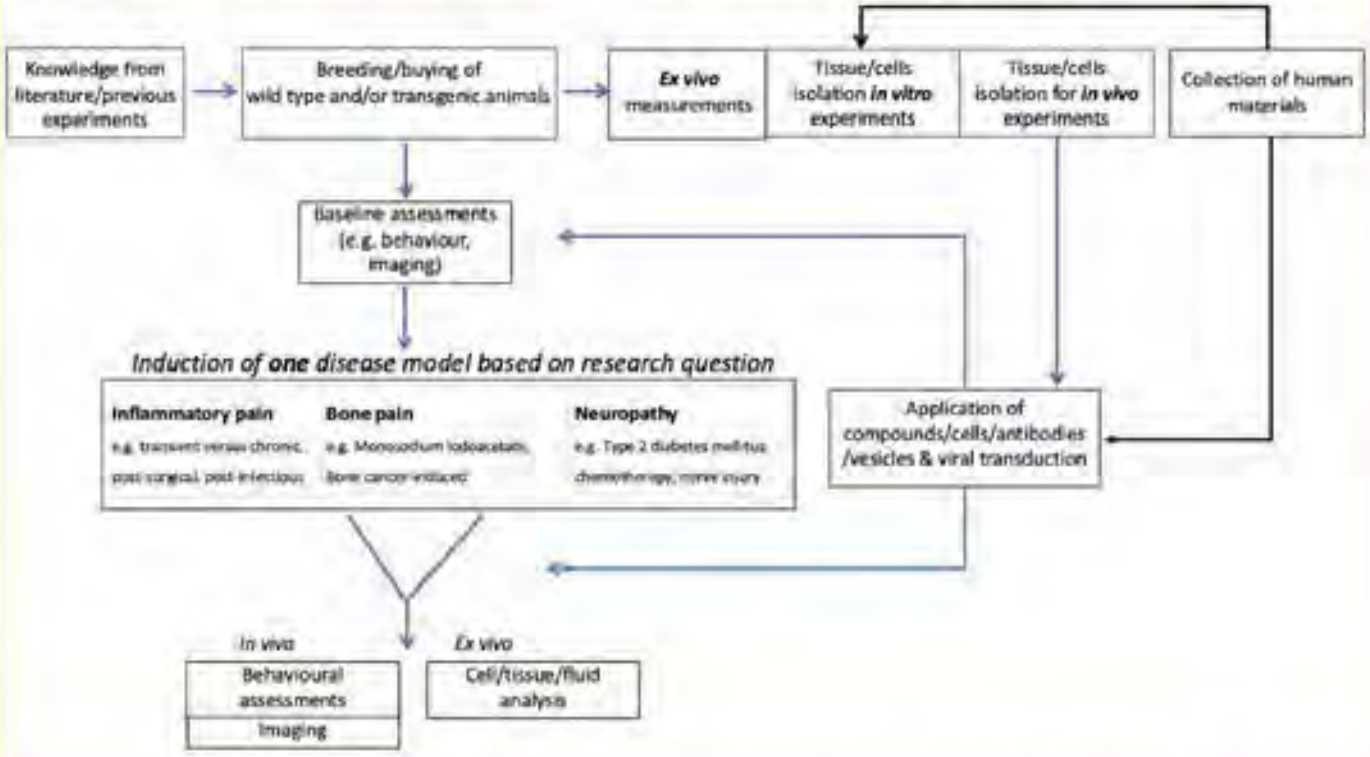
3.4 Strategy

3.4.1 Provide an overview of the overall design of the project (strategy). If applicable, describe the different phases in the project, the coherence, the milestones, selection points and decision criteria.

To fully understand the complex interplay between the nervous system and immune system, in both pain resolution and its persistence, we cannot do without animal research. Furthermore, evaluation of proof-of-concept that targeting a candidate interferes with this interplay between the nervous and immune system and can resolve chronic pain is currently not possible without animals. Where possible, we will use human patient-derived materials to investigate factors/pathways to study *in vitro* and *in vivo*. For example, we will use co-cultures of neuronal and immune cells to assess whether specific factors regulate intercellular communication involved in chronic pain. Conversely, insights gained from in vivo experiments will inform the development of cellular assays and help validate therapeutic targets *in vitro*. This bidirectional approach ensures that findings are both mechanistically grounded and translationally relevant.

In figure 2, we show a general workflow of the strategy of our project, showing the use, continued use, and fate of animals in this project. The purpose and use of animals are indicated with the arrows. Based on knowledge from literature and previous experiments, we will choose the appropriate model/method for our experiments. Only one model will be induced in each animal. We will use animals for ***ex vivo* assessments** (e.g. blood collections), ***in vitro* experiments** (e.g. DRG culture), the **isolation of donor cells/materials for *in vivo* experiments** (e.g. injection of primary macrophages), or we will do **in vivo experiments** in which we induce a pain models in either wildtype or transgenic mice. Next to material obtained from donor mice, we could also apply compounds and antibodies or genetically manipulate the mice in order to evaluate the effect of a target on the pain model. Human material (e.g. patient-derived autoantibodies) can also be used in the animal in vitro and in vivo experiments. However, those substances will not be applied in the same experiment but are used in separate experiments. The choice for an in vivo or in vitro experiment will be based on the level of previous knowledge and research question. In appendix 1 and 2 we specified our strategies.

Figure 2: *General workflow of this project, showing the use, continued use, and fate of animals in this project.*



For each objective we clarify how our step-by-step approach is aimed to achieve our final goal with clear go and no-go moments to reduce the number of animals needed for each objective (figure 3). We show the most important decision moments to reduce animal numbers and discomfort. In general, we decided for each objective which animal models we will use to investigate the main research questions. We selected specific animal models based on results from our previous experiments and based on information from literature indicating which models are most relevant to be able to answer the specific research questions.

For **objective 1**, we aim to dissect neuro–immune interactions that drive resolution versus chronification of pain. Targets (e.g., complement, IgG, hIAPP) are determined by using literature, patient data and previous *in* and *ex vivo* data (e.g., transcriptomic data to identify factors linked to pain resolution, changes in intracellular pathways and neuro-immune communication)

Next to *in vivo* experiments, this objective will be supported by *in vitro* experiments (with cell lines or mouse- or human-derived cells) to study the effects of neurons on immune cells (and vice versa). *In vitro* experiments will be prioritized when we want to know the direct effect of a specific factor on immune cell polarization and/or neuronal

activity, as we can run these assays without interference of other cell types. Cells can be genetically modified to study a specific target and/or treated with substances such as inflammatory cytokines to mimic inflammatory pain or proteins that can cause neuronal damage (IAPP). In turn, antibodies, extracellular vesicles or cytokines can be tested as a suppressor or activator of neuronal signaling.

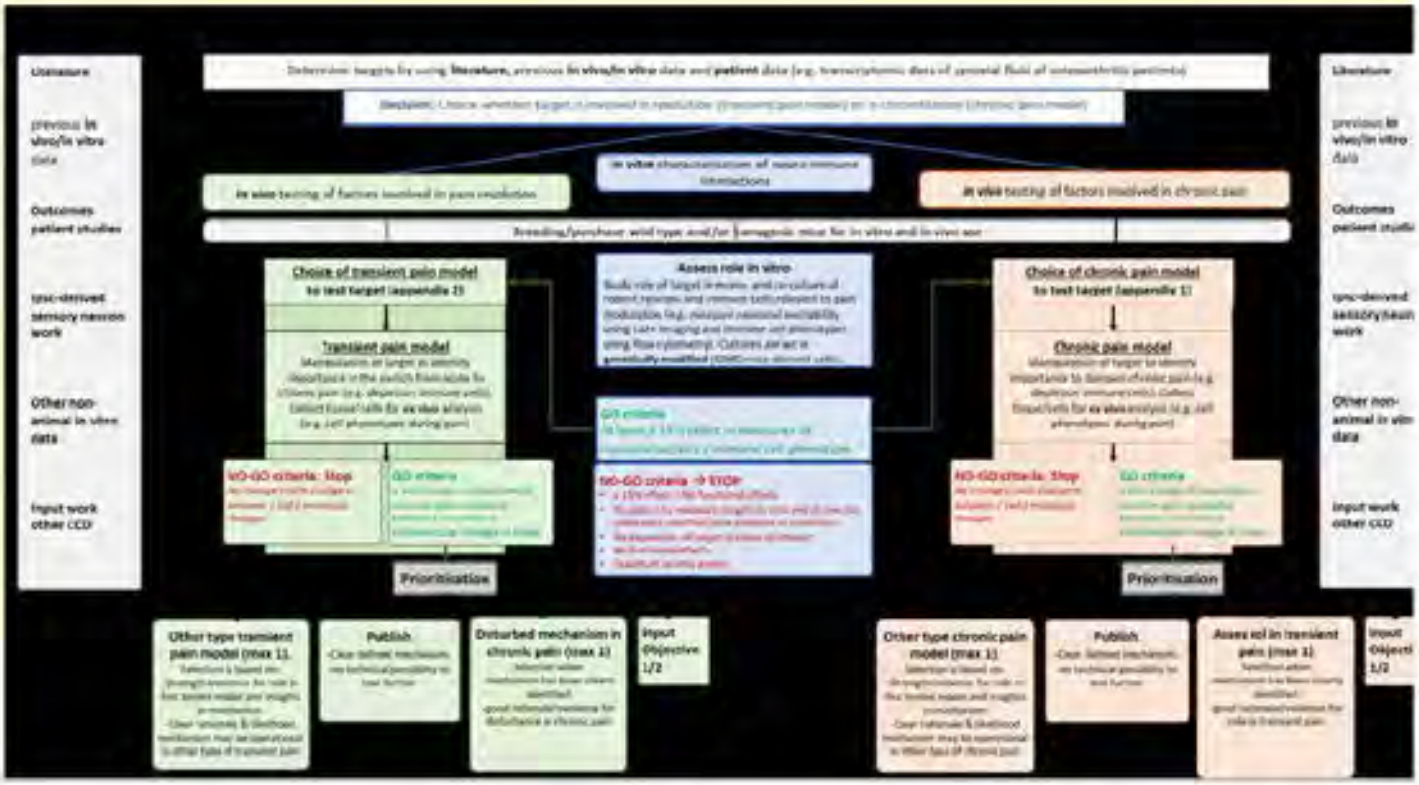
For example, we will employ an in vitro experiment to investigate whether reduced expression of a chemokine (e.g. CXCL11, myostatin) improves the polarization and attraction of anti-inflammatory macrophages to cultured sensory neurons. Based on these results we will determine whether promoting expression of this chemokine has "a go" to be further investigated in our pain models. Go moment is made when we have identified a significant alterations of 15% in sensory neuron activity and/or immune cell phenotype. If we do not see these alterations, it is a 'no-go", and these candidates will not progress to animal pain models.

To test neuroimmune interactions on pain behavior, we need *in vivo* experiments. We will use both transient (resolving; appendix 2) and chronic (non-resolving; appendix 1) pain models to compare physiological versus pathological neuro-immune responses. By understanding the crosstalk between neurons and immune cells during different timepoints in of the pain model, we expect to identify mechanisms important for the induction, resolution and maintenance of pain. A transient pain model enables us to test which specific immune cell/factor is regulated and required for pain resolution. Conversely, chronic pain models will help to investigate which neuroimmune factors are required to maintain pain. The decision on which pain model is used is based on the specific research question and knowledge from literature, patient data, our previous data, and in vitro work. If we do not have indication which model to test based on literature etc., we will start our experiments using a transient pain model (appendix 2). For example, upon identifying a specific target (e.g. chemokine) that is dysregulated in the synovial fluid of osteoarthritis patients, we will study this target in a chronic osteoarthritis model. Conversely when we identify a factor associated with pain resolution, it will be tested in a transient pain model. When a sufficient effect is observed, defined as a significant 30% change in pain-associated behavior when we inhibit this target, this is considered as a go. A change of <30% is considered as a no go. We selected 30% because it is a similar percentage used in the clinic to consider clinically relevancy (PMID: 32520773/ 11690725). After a go, we will prioritize what the next step is. These next steps consist of follow-up due to a new research question (input for objective 1 and 2), decision to publish, or in case the mechanisms has been identified and there is based on literature a good rationale we will test whether the mechanism is broader applicable by testing it in another pain category, or test whether the proposed mechanism is dysfunctional in transient pain (when target was tested in transient model) or operational in transient pain (when target was tested in chronic pain model) (see figure 3).

To give an example base[redacted]d findings, we identified that pro-inflammatory macrophages in the DRG contribute to OA-pai[redacted]With an additional experiment we identifie[redacted]ough which macrophages were recruited and how they gained their pain-maintaining phenoty[redacted]Given that at that time we had a clear insights in the mechanism, we then prioritized to test whether macrophages depletion in another type of bone pain also impacted pain (manuscript in preparation).

Ex vivo material from experiments will be used to analyze phenotypes of immune cells and neurons (e.g., metabolomics on DRGs, microscopy on tissue slides, use of biosensors). Only when there is a behavioral effect in a chronic pain model (30% decrease of pain-associated behaviors), a Go decision is made to study objective 2.
In **objective 2**, we will test for **proof-of-concept of targetability of defined neuro-immune mechanisms in chronic pain.** We want to determine whether an identified target offers broad therapeutic potential for chronic pain or is specific to certain condition. Importantly, the aim is not to develop a specific drug, but rather to identify and characterize potential therapeutic strategies and mechanisms that could inform future pain interventions. For each pain type, **one** representative model will be selected for target validation. For example, if a candidate target is identified in the MIA model of osteoarthritis, it will be verified in an independent model of bone-related pain. The choice of validation model will be guided by scientific literature, biological relevance, and the expertise or preferences of project collaborators. To therapeutically manipulate the neuro-immune environment and support resolution of pain, we will test a range of intervention strategies designed to either **enhance endogenous pain-resolving mechanisms, or block processes that maintain chronic pain.** These strategies will be applied only in **chronic pain models.** Examples of therapeutic strategies include the activation of inhibitory immune receptors, and administration of antibodies to block e.g., complement or IAPP. Initially we will focus on targeting complement and

IAPP, but other leads from objective 1 may feed into objective 2. When there is no effect on pain behavior, experiments regarding this specific therapeutic intervention will be stopped in other chronic pain models. When the intervention results in improved pain outcomes (>30% pain relief compared to control), it will be tested in a one model representing a different pain category. This will allow us to evaluate the broader applicability of the intervention across distinct pain etiologies. *In vitro* experiments (with cell lines or mouse- or human-derived cells) will be used to test whether the specific intervention induces functional cellular changes relevant to pain modulation. These assays will assess effects on neuronal excitability, immune cell activation states, cytokine release, and neuro-immune signaling dynamics to determine the potential of the intervention to support pain resolution or inhibit pathological sensitization.



2. Proof of concept of targetability of defined neuro–immune mechanisms in chronic pain

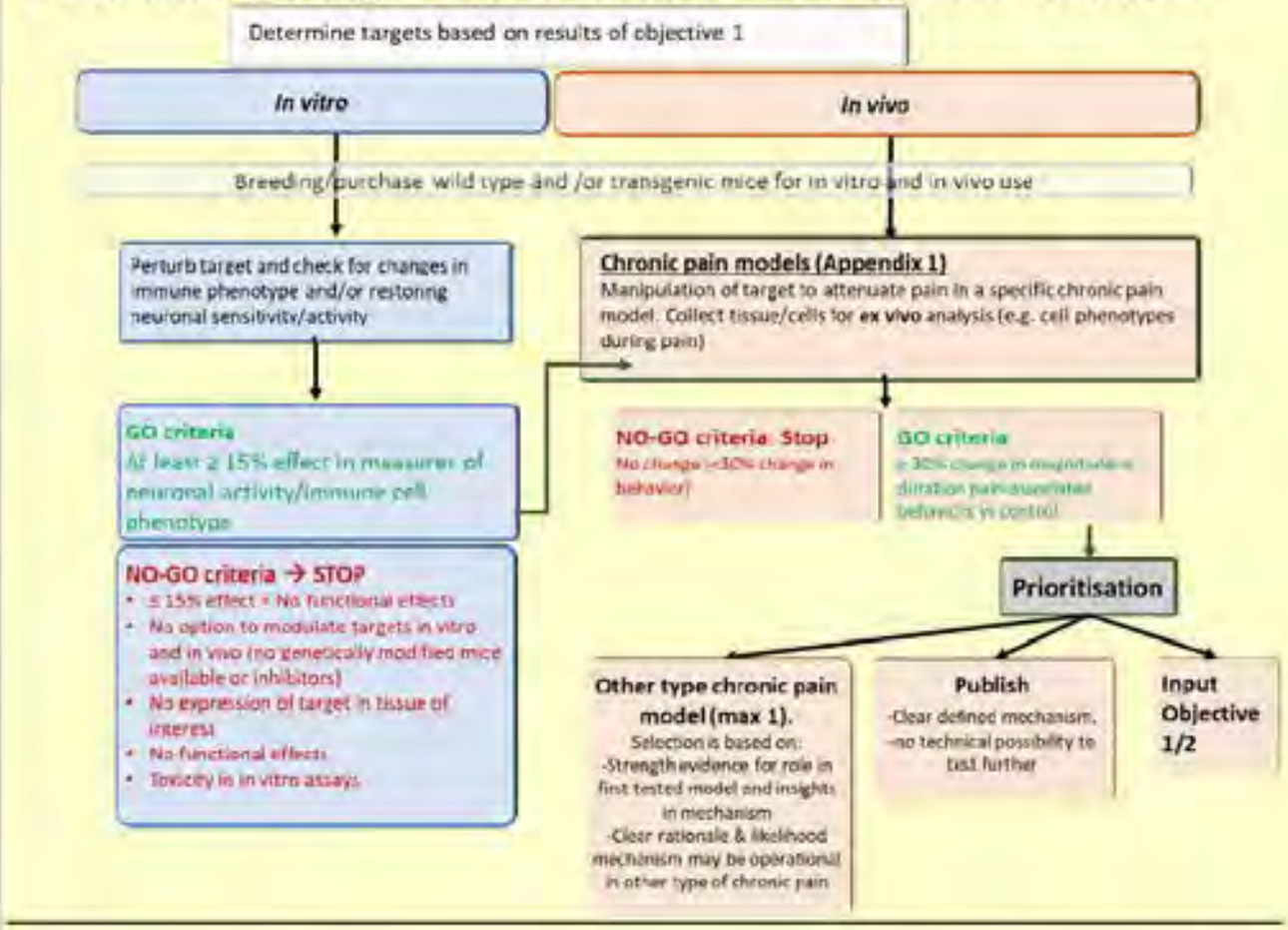


Figure 3: *Project strategy with go no-go criteria. The use of in vitro and in vivo experiments to investigate how neurons and immune cells communicate to regulate pain. The assessments used to measure pain are described in Appendix 1. For in vivo we use 30% cut-off because we want to have clear effect sizes and given that a similar percentage is used in the clinic, where pain VAS score have to reduce with 30% or 2 VAS points to consider it clinically relevant (PMID: 32520773/ 11690725). For the in vitro assays, we take a smaller percentage because in these studies we only asses one aspect, e.g. neuronal functioning, effect immune cells, whilst in vivo it is the combination of all. Based on our previous experience, we observed that e.g. co-culturing neuronal cultures with anti-inflammatory macrophages reduced neuronal activity more subtle. So based on these observations we determined >15% as a "GO" for in vitro experiments. If there is clear evidence that factor X is involved in chronic pain, we directly start with in vivo measurements. However, in some experiments we first start with in vitro assessments (see * above in vitro models) to investigate whether targeting factor X could prevent neuronal damage. For objective 2, we start with in vitro to test which e.g. antibody is most effective to prevent hIAPP aggregation and neuronal damage. From these in vitro results we prioritized the best effective antibody to test in vivo.*

A practical example, we aim to investigate whether an inhibitory receptor (e.g., LAIR) is important for communication with the nervous system and thereby regulating inflammatory pain because we found it was strongly upregulated in transient inflammatory pain, suggesting a putative role in pain resolution. Therefore (see figure 3), we will start testing its role in an transient model, in this case inflammatory pain (low dose carrageenan; appendix 2) as we identified changes in expression in this type of pain. To do this, we will use genetically modified mice that lack LAIR and test whether inflammation-induced sensory hypersensitivity (von Frey, Hargreaves, dynamic weight bearing test; appendix 1) persists in the LAIR knockout mice compared to wildtype mice. If we identify a phenotype

according to criteria as displayed in figure 3 (if we do not, it is a NO GO), this will in this case be used as input for objective 1 for another research question. In vitro and based on literature we know that LAIR is expressed in various immune cells: Macrophages or T cells. Therefore the next question is, is LAIR on macrophages or T cells important for pain resolution? We will then again use the same model, transient inflammatory pain in LAIR deficient mice, but now do intrathecal injections to administer T cell or macrophages that do express LAIR, to see if we can rescue the phenotype. To do this, we isolate T cells/macrophages from mice listed in Appendix 3 (which undergo no other procedures). Now we can ask again the question, do we know sufficiently about the mechanisms and can we publish? Or given the discovered mechanism do we want to know if these same mechanism govern resolution of transient chemotherapy-induced peripheral neuropathy (CIPN) because literature suggest that T cell play a role in this transient CIPN. Or do we want to see whether in a chronic inflammatory pain model this mechanism fails, or whether this is sufficient evidence to test whether targeting LAIR reduces chronic inflammatory pain (objective 2)?

3.4.2 Provide a justification for the strategy described above.

To justify our strategy, we propose a basic outline of the principal components:

Generation, breeding and maintenance of genetically altered animals
We will use wild type mice, transgenic mice with a gain of function mutation, transgenic mice using Cre-loxP system to delete genes in specific cells or global knockout mice and examine the consequences of gene alteration for pain perception and inflammation. Genetically altered animals will be generated and acquired from external sources or are already present at the animal facility. New combinations of Cre - and LoxP lines may result in new conditional knockout mice. Mice will be used to establish a breeding colony, and offspring will be marked by ear punching; the ear tissue will be used for genotyping. Mice with the appropriate genotypes will be used either for in vivo experiments or killed for ex vivo use/analysis. In addition to the use of transgenic mice, we will apply different techniques to alter gene expression levels in vivo, such as administration of antisense oligodeoxynucleotides and/or viral transduction systems to knockdown/overexpress our 'gene of interest'. If we need a specific genetically modified line with discomfort profile to answer our research question, we will monitor or breed these mice under the appendix 4 "Breeding of genetical altered mice discomfort profiles".

A subset from all the mice we will breed or buy, will be directly killed for in vitro experiments to obtain knowledge on how compounds, immune cells or genes influence cells relevant for pain perception. These experiments either 1) provide direction for the approaches for our in vivo experiments (e.g. compound selection) or 2) provide more mechanistic insights to explain our in vivo findings (e.g. which receptor is involved in the effect of a compound, gene, or immune cell on sensory neuron properties). Mice will also be used to obtain donor immune cells for adoptive transfers to elucidate their role in regulating different chronic pain states (see appendix 3). Within this CCD we want to reduce the number of mice for culturing neuronal cells, since we are setting up a facility to culture human inducible pluripotent stem cells (iPSC) that can be differentiated to the "cell of interest". This may increase translatability of our findings to the clinic and reduce animal numbers.

Pain models
Studying the mechanisms of clinically relevant pain states requires the use of animal models having some sustained tissue injury/inflammation. Without such models, it is not possible to fully understand the pathophysiology of chronic pain states. Although animals cannot verbally communicate pain, withdrawal reflexes and exhibition of behaviours indicating defense and discomfort (nocifensive behaviour, such as guarding and shielding), are reproducible and consistent across pain models. These behaviours are attenuated by analgesics, indicating that they are a reliable measure of pain in animals.
It is clear from our own research as well as research from other institutes that there is not one common pathway for all types of pain and therefore we will use different pain models that we have experience with, including:

- Inflammatory pain
- Bone pain
- Neuropathic pain

We have chosen specific models that we will mainly focus on, based on literature and our own findings. These different models are more extensively explained in appendix 1 and 2.

Measurement of pain and sensory thresholds and incorporation of 3R

We will examine how genetic modifications or administration of different compounds affect the performance of the somatosensory system in terms of pain behaviour. We will use different methods (see table 3 in appendix 1), but the most common methods that are used are Von Frey and Hargreaves, to assess mechanical and thermal sensitivity, respectively. Other assessments will be used depending on the specific objective and/or pain model (see appendix 1 and 2). For example, for unilateral pain models dynamic weight bearing tests will provide additional information. We will use videotaping to assess grimace scale as in this particular type of pain the grimace scale is useful. The cold sensitivity test is useful after chemotherapy because chemotherapy affects cold sensitivity, whilst in other models, cold sensitivity is not affected. For some long-term models (> 2 weeks) we will not measure the mice daily, but once a week or once a month. This will be mentioned in the work protocol for that specific pain model. Additionally, cognitive functions might also be affected during pain (examples: brain fog in CIPN and IgG-derived from long-COVID patients). Therefore, in some experiments, we want to use cognitive tests such as the Puzzle Box Test (PBT). Moreover, we will use cognitive wall test (for memory/learning) in our home-cage monitoring system to measure cognitive changes, but also alterations in social interactions and naturally behavior like digging, rearing, locomotor activity (Hasriadi et al., 2021) since these behaviours are affected during chronic pain and could be reverted by analgesics. This way, we get more translationally relevant data using the same animals (reduction), with less stress because we don't have to take them always out from the home cage (refinement) to measure behavior.

We have selected appropriate models to cover clinically relevant types and pathologies of pain, as it is clear from previous research that there is likely not one common pathway for all types of chronic pain conditions. For that reason, we have chosen models that are 1) validated and widely accepted and 2) cover those types of pain that represent the largest clinical need for better understanding and development of new therapeutics.

In most mice, a pain model will be induced. Prior to the induction, pain-like behaviours and sensory thresholds are measured to obtain baseline values, whilst measurements after the induction will provide valuable information on the course of the pain models. In most of these animals, we will apply strategies to modify pain, either by administering compounds or by modulating gene expression or immune cells numbers to be able to provide evidence for involvement or therapeutic efficacy in a specific type or multiple types of chronic pain. A small proportion of the animals will not undergo a strategy to modify pain but will be directly used for ex vivo analysis of changes in cell and gene regulation (appendix 3). This information will tell us where, when and how we should modify a gene, or immune cell subset, or apply a compound.

- The selection of which specific pain model will be used is based on available knowledge and experimental strategies. We will use specific types of pain for each of the three specific objectives as is explained in section 3.1 (background).
- The compounds and concentrations used will be determined based on in vitro and in vivo evidence for a role in the regulation of cells important in pain perception and inflammation, evidence in literature including information on dosing, selectivity and/or mechanism of action.
- The choice of applying gene modification will be based on the evidence (from in vitro and in vivo experiments) that points towards that specific gene, availability of transgenic or knockout mice or based on whether gene modification strategies (e.g. viral mediated gene expression) would be applicable to address the specific research question.
- The selection of targeting specific immune cells is based on our previous research and will be further directed on the basis of evidence in literature pointing towards a relevant role of the specific immune cell subsets, based on, for example, their presence at anatomical sites linked to pain processing.

After induction of a pain model or after a modification (gene/immune cell), animals will be killed (at different time points) to elucidate changes in gene and protein expression profiles due to the induction of pain or after targeting specific pathways/genes. In addition, blood will be sampled at different time points for measurement of cytokines or other neuro-immune factors. All these approaches will directly meet our objectives in providing data on mechanisms, drugs or strategies that might be useful in treating inflammatory, bone and neuropathic pain in humans. Since one animal can undergo several procedures, we estimated the maximal cumulative discomfort an animal may experience (see appendix 1 and 2).

In some cases, we will not continue with the planned procedures, for example:

- If any animal is seen to be having an extreme reaction, induced or not by our models, which are more severe than the estimated maximal cumulative discomfort, this animal will be killed humanely. The precise expected adverse

reactions which will be considered as a reason for humane endpoint are specifically described in appendix 1 and 2.

- If any genetic modification or protein modification results in exceeding the expected pain thresholds, these animals will not be used for further pain inducing models, but they might be used for testing of putative analgesic compounds.
- If any genetic modification does NOT have any effect on pain measurements before or after induction of any pain model, these animals will not be bred and used anymore. Finally, if obtained results indicate that there is one generic mechanism that drives chronic pain, we will decide to continue with only one animal model per objective. We will choose the animal model with the largest treatment effect and if possible, with the least expected discomfort/unwanted side effect, and/or the animal model for the disease with the largest clinical/societal impact.

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

Serial number	Type of animal procedure
1	Chronic pain models & measurements
2	Transient pain models & measurements
3	Obtaining donor cells & cells for ex vivo analysis
4	Breeding of genetically altered mice with discomfort profiles
5	Click or tap here to enter text.
6	Click or tap here to enter text.
7	Click or tap here to enter text.
8	Click or tap here to enter text.
9	Click or tap here to enter text.
10	Click or tap here to enter text.

Centrale Commissie Dierproeven

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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMCU, Utrecht University
1.3	List the serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number: 1 — Type of animal procedure: Chronic pain models & measurements

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.

Our research has two goals:

1. Dissect neuro–immune interactions that drive resolution versus chronification of pain

2. Proof of concept of targetability of defined neuro–immune mechanisms in chronic pain

For goal 1, we will use both transient (appendix 2) and chronic pain models. This dual approach is essential, as clinical observations show that while pain resolves in many patients, once the underlying inflammation or tissue damage subsides, up to 30% of the patients continue to experience pain despite resolution of the initial cause (Wylde et al.,2011; Taylor et al., 2010). This highlights a critical gap in our understanding of pain chronification and underscores the need to investigate the mechanisms that sustain pain in the absence of ongoing pathology. Based on our previous work, we hypothesize that changes in neuro-immune communication are a key driver of these persistent symptoms.

For goal 2, we will use only chronic models of pain. By comparing different types of chronic pain (e.g., neuropathic, inflammatory, bone pain), we aim to uncover both shared and distinct neuro-immune mechanisms that contribute to pain chronification. We will focus on **one** type of pain, but depending on the research question and findings we may decide to test our findings in **one** other model from a different pain category to test whether a potential target is specific for one category of pain or also effective for another. In the main document we gave a short introduction about the different types of chronic pain, but we will shortly offer more details on each type of pain, supported with clinical examples;

Inflammatory pain arises from immune activation triggered by tissue injury, infection, or autoimmune processes. Key mediators like prostaglandins, bradykinin, and pro-inflammatory cytokines (TNF-α, IL-1β) directly sensitize peripheral sensor neurons, leading to allodynia, hyperalgesia and spontaneous pain (Pinho-Ribeiro et al., 2017). Chronic inflammatory conditions contribute significantly to the burden of persistent pain. Some examples are;

- ***Inflammatory bowel disease (IBD),*** affects 10.5 to 46.14 per 100,000 individuals in Europe, with up to 70% of patients reporting ongoing abdominal pain (Coates et al., 2023)
- ***Post-surgical inflammation,*** such as after laparotomy, hernia repair, or thoracotomy, results in chronic pain in approximately 2–10% of patients beyond one month post-operation (Lovich-Sapola & Smith et al., 2015).
- ***Post-infectious pain,*** particularly following SARS-CoV-2 infection, is a growing concern, with around 10% of individuals developing persistent symptoms, including pain (post-COVID syndrome). Interestingly, blood and tissue (muscles) analysis from Long-COVID patients indicate mitochondrial and metabolic disturbances (Appelman, et al., Nat Commun, 2024; Guo et al., EBioMed, 2023).
- ***Rheumatoid arthritis (RA),*** affects 17.6 million people globally and is two-three times more common in women than in men (RA collaborators, Lancet 2023)

Neuropathic pain affects millions of people globally and arises from damage or dysfunction in the somatosensory nervous system. It is associated with a wide range of underlying causes (Girach et al., Pain Res Mag, 2019). Peripheral neuropathy is a major cause of neuropathic pain, and affects ~7-10% of the global population (Girach et al., Pain Res Mag, 2019; Baute et al., Curr Treat Options Neurol, 2019). It can result from rare familial or acquired amyloid polyneuropathies, chemotherapy or trauma. Some examples are;

- ***Chemotherapy-induced peripheral neuropathy (CIPN),*** affects 30-80% of cancer patients undergoing treatment and often persists beyond treatment completion. Neurotoxic effects of chemotherapeutic agents trigger immune activation and mitochondrial dysfunction in sensory neurons.
- ***Type 2 diabetes mellitus (T2DM),*** is strongly associated with obesity. The global rise in obesity rates had contributed to a corresponding increase in T2DM prevalence, and up to 50% of T2DM patients develop peripheral neuropathy, a common cause of chronic neuropathic pain (Hicks and Selvin, Curr Diab Rep, 2019). Emerging research indicates that the aggregation of hIAPP may play a key role in neuro-inflammation and diabetes-induced neuropathic pain (Albariqi et al., J Clin Invest, 2023).
- ***Nerve trauma,*** due to surgery or physical injuries can lead to neuropathic pain. The prevalence varies depending on e.g. the type of surgery, ranging from 10-50% for chronic post-surgical pain, while amputations are associated with neuropathic pain in up to 85% of cases (Kuffler, Mol Neurobiol, 2018).

Bone pain may arise due to various conditions, including injuries, mineral deficiencies, osteoarthritis, and cancer-induced bone pain (Breivik et al., 2006).

- ***Osteoarthritis (OA)*** is the most common degenerative joint disease, affecting more than 500 million people worldwide. Due to aging populations and rising obesity rates, the global burden of OA is projected to increase significantly, with an estimated 130 million people affected by 2050 (GBD, Lancet 2021; Hunter et al., 2020)
- ***Cancer-induced bone pain (CIBP)*** is caused by primary or secondary bone tumours. Bone is one of the most frequent sites of metastases, occurring in 30-40% of patients with lung cancer, and up to 80% of those patients with metastatic lung cancer develop CIBP. This form of pain results from a combination of disrupted bone remodelling (imbalance between bone formation and resorption) and direct tumor-induced structural damage to surrounding tissues, including sensory neurons (Mantyh et al., 2014). Although bone pain conditions

have both inflammatory and neuropathic features, the exact mechanisms driving the pain remain unclear (Falk & Dickenson, 2014).

Our group investigated neuro-immune interaction under CCD AVD11500202010805 and demonstrated that e.g., macrophages have opposing roles in different chronic pain models, particularly in inflammatory versus bone pain conditions. These findings underscore the importance of studying specific immune cell subsets across diverse pain etiologies. For example, anti-inflammatory macrophages infiltrate the DRG in transient inflammatory pain models, and depletion of macrophages promoted the switch from acute to chronic pain. Conversely, pro-inflammatory macrophages innervate the DRG and maintain the pain in CIBP and OA-models, [redacted] and OA or could even reverse established OA [redacted] These findings highlight the context-dependent [redacted] types in disease-relevant models. Moreover, both human and mice studies identified various factors in spinal fluid or joints that are associated with altered neuro-immune interactions and chronic pain, such as elevated chemo-attractants (e.g. CXCL11/myostatin) and increased complement factors. Despite these insights, the precise mechanisms and the specific roles of immune cells in chronic pain conditions remain largely unknown.

To address our knowledge gaps, we will employ disease-relevant models to investigate how immune cell phenotypes and immune factors contribute to the persistence of pain. To study this we will use;

1. Specific (Cre-loxP system) and/or global knockout-animals and transgenic animals to affect gene expression
2. Adoptive transfer experiments to modify immune cells
3. Viral systems to overexpress/knockdown genes of interest in specific cells of interest
4. Pharmacological approaches by using compounds/biologics to target immune cells and/or neurons, receptor/ligands or signalling cascades to interrogate their involvement in neuro-immune regulation of pain.
5. *In vivo* and *ex vivo* imaging to assess neuro-immune functions. The genes/compounds that we will use *in vivo* are selected based on result of (previous) work *in vitro*, literature research and findings in patient-derived biological materials.

A general design of an animal experiment is illustrated in figure 1. For each experiment, animals can undergo different protocols/procedures. For some experiments, animals will be killed directly to obtain primary cells for *in vitro* experiments without induction of a specific pain model or strategy to modify pain, those will be separately discussed in appendix 3.

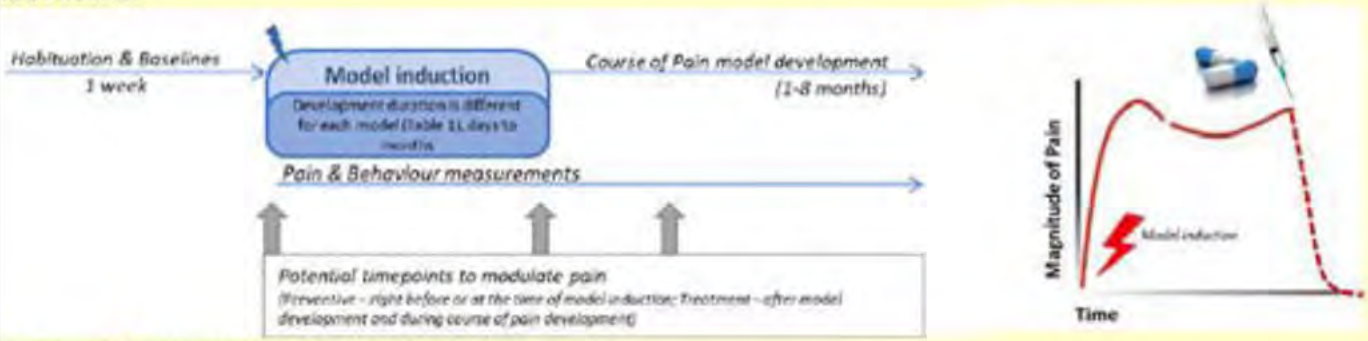


Figure 1: A general design of an animal experiment

Prior to the induction, pain-like behaviors and sensory thresholds are measured to obtain baseline values. Subsequently **one** model is selected from table 1 to induce persistent pain. We can modulate pain at the start of the model, to investigate whether the induction of persistent pain can be prevented. Alternatively we will provide a treatment when persistent pain is established, to test whether we can resolve the pain (figure 1 and 2). Sometimes is one treatment intervention not sufficient to dampen the pain. In this case, we will use **max three procedures** per animal, to secure that the cumulative discomfort is maximally moderate.

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

Each experiment has a general outline as shown in figure 2, with max. 3 procedures to modify genes, signalling cascades, or cells and different moments that we cull mice for e.g. ex vivo analysis. The general outline is detailed below in which we describe 3 experimental options.

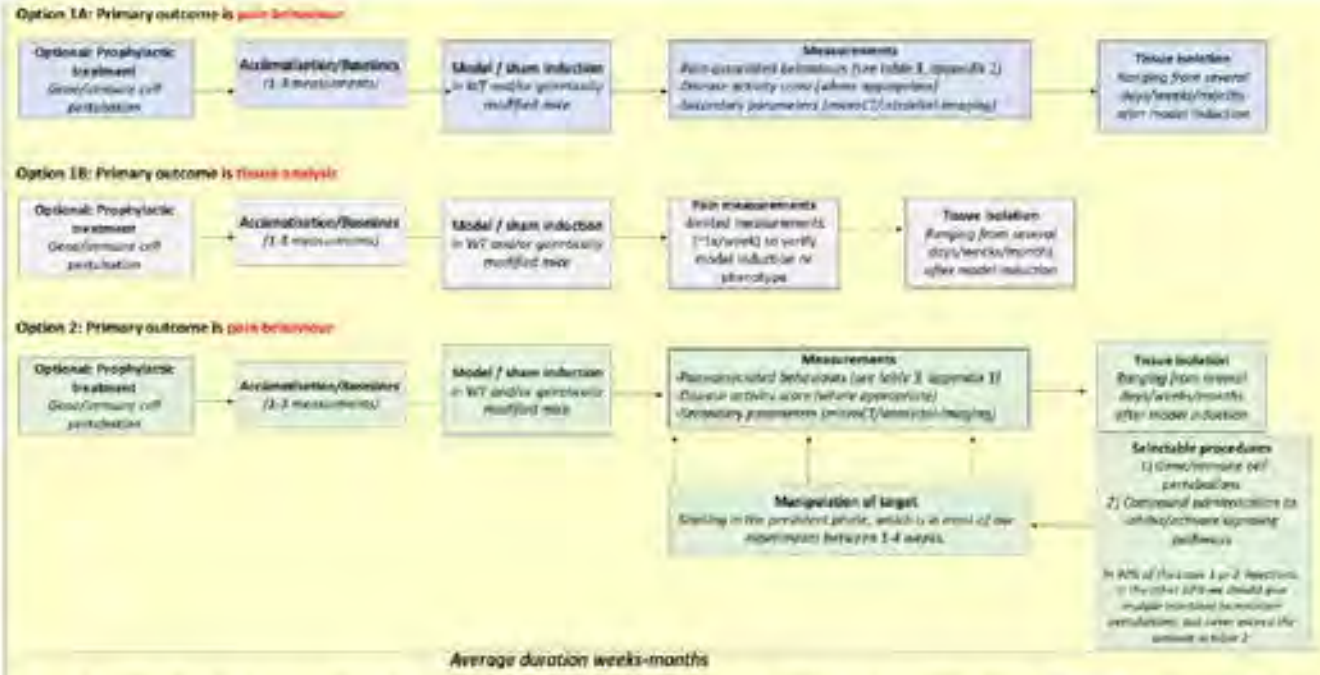


Figure 2: schematic overview of a design of an experiment and optional procedures that can be used for each model. The procedure for model induction is shortly (e.g. intrathecal injection is max 3 minutes), but the consequence is that pain symptoms may develop that persist over a longer time period, ranging from 1 week to several months. However the procedure could also induce pain resolution and thereby stop the pain.

The animal models that we will use (table I) are described below. Depending on the current literature, our previous findings and discussions with our scientific collaborators, we will use **one** starting model for objective 1. For objective 2, we will use in **one** other pain model from the same category as objective 1. By a go-moment we can do once an extra model from another pain category to be able to tell whether that specific mechanism is pain type specific or represents a more common general mechanism. For example, when we test whether enhancing chemokine expression promote infiltration of anti-inflammatory immune cells in the DRG to alleviate neuropathic pain, we begin with a chemotherapy-induced neuropathy model. If enhancing chemokine expression leads to a significant reduction in pain in this model, we will extend our investigation to other neuropathic pain models, such as nerve injury (e.g., sciatic nerve ligation) or amyloid-induced neuronal toxicity (e.g., human islet amyloid polypeptide, hIAPP). This stepwise approach allows us to determine whether targeting a specific chemokine represents a broadly applicable therapeutic strategy for neuropathic pain, or whether its efficacy is limited to specific etiologies, such as chemotherapy-induced neuropathy. In table 1 we describe the models; the dose, route, frequency and max duration. Below the table there is more in-depth explanation of the models.

*Table 1: Different chronic pain models that could be used in mice (except for the groove model which is in rats). The **bold** models are the preferred starting models.*

INFLAMMATORY PAIN

Agent	Dose	Route	Frequency	Max Duration	Anesthesia	Analgesics	Severity
Inflammatory soup	Carrageenan (2% (w/v), 20 µl) or Complete Freund's Adjuvant (100%, 20 µl)	Intraplantar	1x	30 days	No	No	Moderate
Priming model Carrageenan low dose followed 1 week later by inflammatory mediators (e.g., PGE2 or carrageenan low dose)	1% (w/v), 5 µl 1 pg-1µg	Intraplantar	1x 1x	7 days (first phase) + 30 days (second phase)	No	No	Moderate
Patient-derived autoantibodies	~6.5 mg IgG/mouse (260mg/kg)/ 40ug	Intraperitoneal /intrathecal	1x	21 days	No	No	Moderate

NEUROPHATIC PAIN

Chemical-induced pain models							
Chemotherapy-induced peripheral neuropathy	E.g. Paclitaxel 8 mg/kg	Intraperitoneal	4x	30 days	No	No	Moderate
Neurotoxic (amyloid-induced)	40 ug/kg (10-1.000 fg)	Intravenous or intraplantar	1x	30 days	No	No	Moderate
Mechanical-induced pain models							
Spared nerve injury (SNI)	Operation	Ligation Tibial nerve, common peronal of the sciatic nerve	1x	30 days	Yes	Yes, Temgesic	Moderate
Chronic constriction injury (CCI)	Operation	loose silk suture around sciatic nerve	1X	30 days	Yes	Yes, Temgesic	Moderate

BONE PAIN

Cancer-induced bone pain (CIBP)	Tumor cells in 10-100 ul PBS/medium	Injected in femur or tibia	1x	30 days	Yes	Yes, Carprofen	Moderate
Monosodium Iodoacetate (MIA)	5 µl of (2-8 mg/ml MIA)	Intra-articular	1x	30 days	Yes	No	Moderate
Groove	Cartilage damage with yellow needle (30G)	Intra-articular	1x	24 weeks	Yes	Yes, Carprofen	Moderate

Explanation of the approached models

Based on our extensive experience with the models described below, we can reliably classify all of them as involving moderate discomfort in accordance with the EU Directive 2010/63/EU and Dutch legislation. This classification is supported by previous use, observed animal responses, and established literature. The choice of which model to use is mainly dependent on the research question and on existing knowledge/data, which will be further explained below by each specific model.

Inflammatory pain models

Carrageenan (seaweed extract) and Complete Freund's Adjuvant (high dose)
Indicated inflammatory agents will be injected in 1 hind paws. Although both model are induces moderate discomfort, our preferred model is the carrageenan model as it induces less discomfort (pain). The volume of injection is limited to 20ul reduce discomfort. Studies have shown that exploratory behaviours and stress coping strategies do not changes after an intraplantar injection of CFA (in this case 2,5 more CFA is injected than what we use). https://pmc.ncbi.nlm.nih.gov/articles/PMC8119306/. In these models we have observed that the maximum reduction (at day 2) in weightb[redacted]w is 25% compared to baseline and within a week it is max 15%, so they still use the hind paw[redacted] some cases for ex vivo analysis of tissue (e.g., sensory neurons innervating the affected area), the amount of obtained tissue (unilateral) is not sufficient, whilst when the model is bilateral, we will obtain sufficient material from 1 mouse. When injected bilaterally we will limit the number of other strategies to max 1 and reduce the number of pain measurement to max 3 to reduce the added discomfort by these procedures. In previous work we have seen that mice still walk normally when injected bilaterally. A high dose of CFA will be our first choice model to induce persistent inflammatory pain, since this model is general used in the "pain-field" and therefore several omics-data sets are online available. This helps to test whether our "gene/protein of interest" is affected (PMID: 27103637/32810432) in CFA-models, and whether altering the expression or linked pathway is a novel therapeutic option. High dose of carrageenan is often used as verification model

Priming model
This model represents a model where acute pain transitions to chronic pain, so endogenous pain resolution pathways fail. This will allow us to test if a certain intervention can prevent or revert this transition (Reichling & Levine, Trends in Neuroscience, 2009). In this model, mice will receive two intraplantar injections; first with carrageenan (or vehicle). The carrageenan will induce transient hypersensitivity that typically resolves within ~7 days but causes molecular changes which makes neurons more hypersensitive to a subsequent inflammatory stimulus (called hyperalgesic priming). This hyperalgesic priming is followed by another intraplantar injection after 1 week in the same paw, with an inflammatory mediator (PGE2 or again carrageenan) and this induces prolonged inflammatory hypersensitivity (>2 weeks) whilst normally this injection only induces hypersensitivity for 24 hours (PGE2) or ~7 days (carrageenan). We will use this model to specifically understand the molecular mechanisms contributing to the transition from acute to chronic pain. For example a low dose carrageenan induces transient pain (see appendix 2), however when the pain is [redacted] we give again a single low dose carrageenan, the acute pain transitions into chronic pain (PMID: [redacted]9922165).

Autoantibodies-induced pain
For this purpose we will inject autoantibodies from patients into mice. Recently, we observed that autoantibodies from long-COVID patients induces symptoms in mice (e.g. chronic pain, reduce locomotor activity, Chen et al., 2024, BioRxiv). In the upcoming years, we will investigate several patient groups, including adults and children with long-COVID, post-herpetic neuralgia patients, but also patients with chronic fatigue syndromes (ME/CFS) which often develops after a bacterial/viral infection. Our group as well as other research groups have observed increased markers of neuroinflammation in the blood of these patients. We will investigate whether autoantibodies derived from blood contain factors that promote neuro-inflammation that can be linked to neurological symptoms (e.g. pain, fatigue, cognitive impairments). We aim to identify which antibodies (single or pool of antibodies) are causing these symptoms, by injecting them into mice. We will use this model to mechanically understand how auto-antibodies cause chronic pain. We use this model if patient-material is available and if there is evidence from literature/previous data that autoimmunity is linked to the disease.

Neuropathic pain models
From the neuropathic models, we mainly will focus on hIAPP and CCI, since there is clear evidence that the immune system is strongly involved in these models. The other models could be used as verification models.

Chemotherapy-induced neuropathy
Neurotoxic effects of chemotherapeutic agents trigger immune activation as well as mitochondrial dysfunction in sensory neurons. Chemotherapy-induced pain is developed following the administration of chemotherapeutic compounds such as Paclitaxel or Oxaliplatin, via intraperitoneal or intravenous administration. All these chemotherapeutics are well known for their neuropathy inducing side effects (Lees et al., Eur J Cancer, 2017; Hu et al., Brain Behav Immun, 2018; Makker et al., Plos One, 2017). The dose administered is based on our previous studies and literature, or is determined in dose-findings studies.

Neurotoxic (amyloid-induced)
In Type 2 Diabetes (T2DM), patients develop neuropathy in approximately 50% of cases. Amyloid deposits of human islet amyloid polypeptide (hIAPP) and associated tissue dysfunction are found not only in the pancreatic islets, where hIAPP is produced by β-cells, but also in the heart, kidneys, and brain (Gong et al., 2007; Liu et al., 2016; Jackson et al., 2013). [redacted]hat our T2DM mouse models (hIAPP and hIAPP/ObOb mice) also develop peripheral neuropath[redacted]his model consists of transgenic hIAPP mice mated with or without C57Bl/6J-

ob/Ola/Hsd (ob/+) to generate transgenic hIAPP mice with or without obesity (Ob/Ob). In upcoming experiments we will predominantly use hIAPP mice (without ob) to investigate how hIAPP disrupt neuro-immune interactions. Furthermore, we inject hIAPP in mice both intravenously and intraplantarly, allowing us to investigate its local and systemic effects on pain development.

Mechanical induced neuropathy
We employ models that mimic clinical scenarios of neuropathic pain, such as nerve damage or nerve ligation, in which a peripheral nerve (e.g., the sciatic nerve) is carefully tied off;

Spared nerve injury
Spared nerve injury (SNI) is performed as described previously (Decosterd and Woolf, Pain, 2000). Briefly, under 2% isoflurane anesthesia, the sciatic nerve and its three terminal branches are exposed carefully with minimal damage. Leaving the sural nerve intact, the common peroneal and the tibial nerves are tightly ligated with 6-0 silk surgical sutures and cut 2 to 4 mm distal to the ligation (see figure 3). Sham controls undergo anesthesia, incision, and exposure of the nerve only.

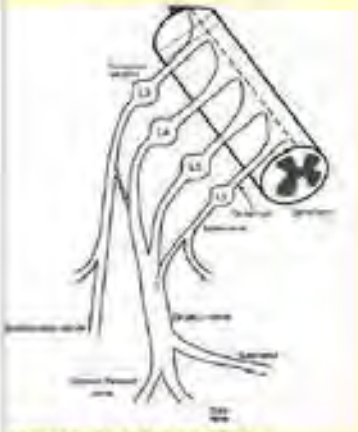


Figure 3: SNI model

Chronic constriction injury (CCI)
Chronic constriction injury is performed as described by Bennett et al. (Pain, 1988). Briefly, the sciatic nerve is exposed in anesthetized animals above the femoral joint. One loose silk suture knot and 3 cat gut sutures are applied to the sciatic nerve, with intervals of 1 mm (see figure 4). The skin is then clamped closed.

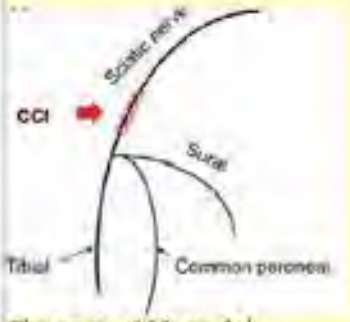


Figure 4: CCI model

Bone pain models
For the bone pain models we will mainly use the MIA model. The MIA OA-model we used in 80% of our research on OA the last 5 years, since it is well described/documented in pain research in mice. This model is more inflammatory-driven. The groove model is less inflammatory, but more cartilage damage-related. The groove model is only feasible in rats (for now), so if we want to use genetically modified mice (e.g. without complement C2), than we need to use the MIA model as starting model.

CIBP
We will choose this model if we want to mimic cancer pain caused by bone tumours. To induce the CIBP model, tumor cells (such as murine lung carcinoma cells) are injected into the marrow space of the femur or tibia while the animal is under anesthesia (Hansen et al., Pain, 2011; Wang et al., JCI, 2020). A superficial incision is made near the knee joint to expose the patellar ligament. A 25-gauge needle is inserted at the intercondylar notch of the femur into the femoral cavity, and tumor cells are injected. To prevent tumor invasion into adjacent tissue, the injection site is sealed with a

bone restorative material (sterile bone wax). The skin is then sutured using 4-0 Ethicon Vicryl rapid suture, and Xylocaine gel is applied to the wound for post-surgical analgesia. Sham-operated control mice undergo the same procedure but receive either 10 µL of medium alone or heat-inactivated tumor cells. Bioluminescence imaging and/or X-ray analysis are performed over time to monitor tumor growth and bone degradation.

MIA

To mimic osteoarthritis in mice we will predominantly use the monoiodoacetate (MIA) model, which will chemically cause cartilage damage. A single intra-articular injection of MIA is administered in the knee of the mouse, while the animal is briefly under anesthesia. Knee joints are shaved, flexed at a 90° angle and 10 µl of 10- 100 mg/ml MIA in sterile saline (0.9%) or control saline are injected through the infra-patellar ligament into the joint space of the knee using a 30-gauge needle (Pitcher, J Vis Exp. 2016). We provided the IvD a representative video of behavior of animals in which the MIA model was induced 8 days earlier (videotaped in homecages). Although the animals experience ongoing pain, they still engage in exploratory and grooming behaviors and use the running wheel, suggesting that this model can be classified as "moderate".

Groove

With the groove model we will surgically induce osteoarthritis. For the groove model, we will use **rats** since it is practically not feasible to perform this model in mice. We will mainly use the rat model as described by Visser et al., 2017 (J Orthop Res) to mimic OA-like pain. Briefly, the tip of a 30G needle was bent and used as surgical tool to apply five longitudinal grooves on the articular cartilage of the femur or tibia. This will be done in addition to three longitudinal grooves on the non-weight bearing surface of the articular cartilage (see figure 5).

Figure 5 Groove-model

Administration compounds/substances

To investigate and understand the molecular mechanisms of pain, different compounds or control substances (alone or in combination, continuously or intermittently) will be administered by one or multiple routes. A schematic description of the drug administration routes (including information such as maximum frequency, maximum number of administration within one experiment and maximum volumes) are reported in Table 2.

Table 2: Description of drug administration routes, maximum volume and frequency following the international guidelines. In practice we always use less frequencies.

		Mouse			Rat		
Route	**Anaesthesia**	**Max Frequency**	**Max total administration**	**Max volumes**	**Max Frequency**	**Max total administration**	**Max volumes**
Subcutaneous	No	2 x day	20 injections	10 ml/kg	2 x day	20 injections	5 ml/kg
Intraperitoneal	No	2 x day	30 injections	20 ml/kg	2 x day	30 injections	10 ml/kg
Intravenous	No	1 x day	Max 7 injections	5 ml/kg	1 day	30 injections	5 ml/kg
Intrathecal	Yes	1 x day	8 injections	7.5 µl	1 x day	8 injections	50 µl
Intra-articular	Yes	1 x day	5 injections	50 µl	1 x day	5 injections	50 µl
Intraplantar	No	1 x day	5 injections	25 µl	1 x day	5 injections	50 µl
Oral (gavage or micropipette)	No	2 x day	Up to 28 days	10 ml/kg	Max 2 on a day	Up to 28 days	10 ml/kg

The compound and concentration will be determined based on in vitro evidence for a role in the regulation of cells, evidence in literature including information on dosage, selectivity, or mechanism of action. Care will be taken to avoid potential known side-effects at certain concentrations. The route and number of applications will be determined based on inducing the least discomfort but sufficient to block or stimulate a specific pathway at a specific period of time.

Behavioral assessments

For all models we will do behavioral assessment and will use 1 or several tests, depending on the research question with a **maximum of 4 behaviour tests** per day/per animal (table 3). For example, we will test mechanical sensitivity (Von-Frey), thermal sensitivity (Hargreaves), locomotor function (rotarod) and cognition (puzzle box). This combination of tests will allow us to address different behaviours. In this example with multiple assessments, each test will be performed max once a day. The choice of tests depends on the animal model that is being used and the pathology it represents. The animals will be acclimatized to the testing environment to minimize stress and fear during the measurements. In case we measure over longer time points we will spread assessment over different days to avoid multiple measurements on a day; if this batteries of test are performed in a day, each test will be performed only once.

*Table 3: Possible behavioral assessments. Predominantly **two** "black assessments" are used in our experiments. They can be extended with one or two "black or grey assessments", depending on research question (e.g. chemotherapy can induces sensitivity to cold, so cold plate test can be added). *max 7 sessions in a 7 day period, can be at the start of the induction model or during the persistent phase if we just gave a procedure (e.g. to monitor if a compound reduces pain)*

	Method	Stimulus	Maximum stimulus duration	Maximum Frequency*	Species
Evoked-hypersensitivity	Von Frey	Calibrated series of von-Frey hairs ranging from 0.02 to 4g	5 seconds per hair	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse and rat
	PAM device	Increasing squeezing pressure	30 seconds, 3x per limb/animal	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse and Rat
	Hargreaves	Focused light beam (infrared) using a Hargreaves apparatus	Max 20 seconds per paw	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse and rat
	Thermal sensitivity	Cold plate	15 min	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse and rat
Non-Evoked-hypersensitivity	Dynamic weight bearing	No stimulus	5 min	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse and rat
	Place-conditioning test	Administration of a pain relieving compounds	2x 30 minutes	Max 5 days	Mouse and rat
	Grimacing Videotaping	No stimulus	0,5-4 hours	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
	Vocalization (Ultrasound)	No stimulus	1 min	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
	Burrowing assay Videotaping	No stimulus	About 16 hours	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
	Grip strength Videotaping	No stimulus	2_5 minutes	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
Activity & cognition	Puzzle box test Videotaping	No stimulus	5 minutes per trial	Max 5 trials daily, for max 4 days	Mouse
	Homecage monitoring Videotaping	No stimulus	24/7	24/7	Mouse
	Open field, Videotaping	No stimulus	1 hour	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse and rat
	Rotarod/accelerating	Increasing rod speed	15 min	Max 7 sessions in a 7-day period. During the persistent	Mouse and rat

In table 3 we separated the different methods to measure evoked-hypersensitivity, non-evoked hypersensitivity (spontaneous pain), activity and cognition. We shortly explain each method:

Evoked-hypersensitivity

Von Frey
Mechanical threshold will be measured using von Frey monofilaments (Chaplan SR, et al., Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994). In brief, animals will be placed on a wired grid base in Perspex boxes, through which the von Frey hairs will be applied (bending force range from 0.02 to 2 gr, starting with 0.4 gr) on the affected paw. The hair force will increase or decrease depending on the response. Clear paw withdrawal, shaking or licking will be considered a positive response. Ambulation will be considered as an ambiguous response, and the stimulus will be repeated.

PAM-device:
We will employ the PAM device to test mechanical hypersensitivity in mouse and rat knees (Barton NJ, et al., Pressure application measurement (PAM): a novel behavioural technique for measuring hypersensitivity in a rat model of joint pain. J Neurosci Methods. 2007). The device consists of a force transducer mounted on a unit that is worn on the researcher's thumb. The apparatus has a force transducer with a range of 0–1500 g. Animals will be lightly but securely held. The thumb unit will be placed on one side of the animal's knee joint and the researcher's forefinger on the other. A gradually increasing squeeze force will be applied by the device across the joint. The test endpoint occurs when the animal withdraws its limb or shows any behavioral signs of discomfort or distress, such as freezing of whisker movement or wriggling.

Hargreaves:
For the Hargreaves test animals will be placed on a warm glass plate (32 degrees) in Perspex boxes (Hargreaves K, et al., J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain. 1988). A light beam will be pointed at the hind paw to induce heat in the paw and latency time until the animals react will be recorded. The maximum stimulus duration is 20s and the machine will automatically turn off the light.

Thermal sensitivity:
Animals are placed on a metal plate where temperatures range from 0-65°C (Yalcin I, et al., Differentiating thermal allodynia and hyperalgesia using dynamic hot and cold plate in rodents. J Pain. 2009). Latency to first response is recorded (clear paw withdrawal, shaking or licking will consider as positive responses).

Non-evoked hypersensitivity
Dynamic weight bearing:
The automated DWB device consists of a Plexiglas enclosure (11×11 cm for mice, 22x22 cm for rats), a floor sensor composed of 44 × 44 captors detecting pressure variation and a camera placed above the enclosure in order to validate the animal position (Robinson I et al., Use of dynamic weight bearing as a novel end-point for the assessment of Freund's Complete Adjuvant induced hypersensitivity in mice. Neurosci Lett. 2012.). The animals are able to move freely in the enclosure during a total time of 10 min, starting with a habituation phase of 5 min followed by a recording phase of 5 min in which the weight distribution and the video will be recorded. The data will be analysed offline.

Conditioned Place Preference (CPP):
In the CPP, a 3-chamber (A,B, and C) box is used, with the B chamber being a smaller chamber connecting A and C, separated from the neighboring chambers by a divider with an entrance (He Y, et al., Negative reinforcement reveals non-evoked ongoing pain in mice with tissue or nerve injury. J Pain. 2012). The A and C chambers have various patterned walls and floors that are texturally different. On day 1 (preconditioning), animals will be allowed to move freely for 30 minutes, and the time spent in each chamber will be recorded. On days 2–4, the animals will be conditioned with daily intraperitoneal injections of vehicle or gabapentin. In the morning, the animal will be placed for 30 minutes in 1 closed chamber (black room), 1 minute after receiving the vehicle. In the afternoon (3 hours later), the animal will be placed for 30 minutes in the other chamber (white room), 1 minute after receiving gabapentin treatment. On day 5, after conditioning, the animals will be placed in the connecting chamber (chamber B) and allowed free access for 30 minutes to chamber A or C. The time spent in the chambers will be recorded.

Grimacing Videotaping: Animals will be videotaped to analyze facial expression over time that are linked to spontaneous pain, such as orbital tightening, nose/cheek bulge, ear position, etc (Deuis JR, et al., Methods Used to Evaluate Pain Behaviors in Rodents. Front Mol Neurosci. 2017).

Vocalisation:

Systems will be used to measure ultrasounds (e.g. sonotrack system) in a special environment that is closed for outsides noises (Williams WO, et al., Ultrasonic sound as an indicator of acute pain in laboratory mice. J Am Assoc Lab Anim Sci. 2008).

Burrowing assay
A long tube (200 mm length x 72 mm diameter, raised 30 mm from the ground in the frontal sealed at one end is filled with a substrate (cage bedding) and placed in a standard transparent plastic cage without bedding (Deacon RM. Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. Nat Protoc. 2006). Each mouse is placed individually in the cage with the described setup. After 2 hours, the amount of material displaced is weighed and recorded. The reduction in the amount of substrate voluntarily burrowed can be used as a surrogate marker of pain-like behaviour.

Grid climbing test
Each animal is placed on a standard wire lid ((Falk S, et al., Grid-climbing behaviour as a pain measure for cancer-induced bone pain and neuropathic pain. In vivo. 2017). The lid is turned upside down with the animal now hanging from underneath for 2.5 min while being filmed from above. Each film is subsequently analysed in slow-motion and the time spent using the affected leg is recorded manually.

In addition to the pain assessments, we sometimes perform behavioural tests to assess secondary outcome parameters. For instance, rotarod or open field may be employed to assess general motor performance after treatment/experimental strategy to exclude impaired motor behaviour which could affect the behavioural pain assays. Or for some models we want to measure cognitive functions, since we know that these can be impaired after chemotherapy or in patients with post-infectious syndromes (e.g., post-COVID).

Activity & cognition
Puzzle Box Test: Mice are individually placed in an open perspex chamber with a tunnel connecting to a closed/dark chamber (Abdallah NM, et al., The puzzle box as a simple and efficient behavioral test for exploring impairments of general cognition and executive functions in mouse models of schizophrenia. Exp Neurol. 2011). Mice rapidly prefer this dark/goal chamber. The time to enter the goal chamber is measured at 3 levels of complexity (across 3days/8trials): tunnel left open, tunnel covered by bedding material and tunnel covered by a plug. Each mouse performs 3trials per day during 3 consecutive days. Each trial has a maximum of 3 min (training and burrowing) or 4 min (plug), even if the mouse fails to solve the task. All sessions are recorded for later analysis.

Home-cage monitoring:
We purchased a home-cage monitoring system (Noldus). This system allows us to measure 2 mice per cage 24/7 for behavior. The advantage is that we can measure behavior for a long time (instead of 30 min) and also during the night, the time when they often show their natural behavior better. We also need to remove the animals less from their home-cage, which gives less stress/anxiety and should bring less variation in the data (less examiner influences). With this, we don't need the rotarod and open field setup. We only need to do Von Frey followed by monitoring in the home-cage. This also allows us to replace the puzzle box with the cognitive wall in the home cage. For this cognitive test, the mice do need to be individually housed for 8-9 hours. (Cassidy BR, et al., Progressive cognitive impairment after recovery from neuroinvasive and non-neuroinvasive Listeria monocytogenes infection. J Infect Dis. 2025)

Open Field:
Animals will be placed in a cage without bedding. Activity will be tracked by video camera above the cage for 30 min (Kraeuter AK & Guest PC, Sarnyai Z. The Open Field Test for Measuring Locomotor Activity and Anxiety-Like Behavior. Methods Mol Biol. 2019).

Rotarod: Animals will be placed on the rotarod (Vandeputte, C., et al, Automated quantitative gait analysis in animal models of movement disorders. BMC Neurosci 2010). We will apply two different measures. 1: Speed of rotarod will increase from 4 rpm to 40rpm in 300 sec. Latency until the animal falls of or does a 'passive rotation' will be recorded. In total 3 trial will be performed with 15 min at least between trials. 2: 10 rpm for 15 min. Time until animals stop walking will be recorded

Recognition memory:
This test consists in 3 phases: Habituation to arena: animals are placed in the empty arena and allowed to explore for 10 min. The next day there is a training or familiar session: introduction to the arena with 2 familiar objects, return to cage, followed by the replacement of one object with a novel object for the testing session (maximum of 10 min each session). If considering place recognition memory, there is an intermediate session where one of the familiar objects is moved to a new location first and then replaced in this location (Vogel-Ciernia A, & Wood MA. Examining object location and object recognition memory in mice. Curr Protoc Neurosci. 2014).

In addition to the behavioral assessments, we sometimes perform tests to assess secondary outcome parameters. For example, to verify good T2DM induction, we will characterize glucose homeostasis/metabolism (basal blood glucose, HbA1c and insulin levels, ITT (insulin tolerance test) and GTT (glucose tolerance test), degree of inflammation (body temperature and blood cytokine levels), degree of amyloidosis, blood levels of amyloid proteins and bodyweight. In

the models where we inject compounds in the paw, we will measure the degree of inflammation by measuring paw or knee thickness after intraplantar injections. If required to measure compounds in blood, blood will be collected from a superficial vessel (totalling < 10 % of circulating volume) at different time points during the experiment. Furthermore, in bone pain models, we will employ microCT imaging (Int J Mol Sci. 2022:23 (7): 3725) to monitor disease progression in surgical and chemically-induced models (for instance in the Groove model and the MIA model) and to evaluate the effectiveness of potential treatments not only in alleviating pain but also in improving joint damage, by comparing microCT images taken before and after treatment. In addition, for some of the research questions, we perform intravital imaging of the spinal cord, DRG and nerves in anesthetized animals. We image genetically labelled fluorescent adducts for organelle-specific (e.g. mitotracker) or metabolic-specific dyes (e.g mitosox for mitochondrial ROS production) while animals are under anesthesia. Laminectomy will be used to visualize the DRGs, nerves, spinal cord. In vivo imaging will allow us to study dynamic processes that are missed in static analysis.

At the end of all the experiments blood, all relevant tissues and/or organs will be collected for *ex-vivo* analysis to reduce the number of animals required. There is a possibility that new ways for pain assessment are being published or developed in the coming 5 years. We would like to be able to include these models if they have shown to produce more reliable results or are less invasive for the animals.

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

For each experiment, power calculation (with Bonferroni correction if multiple groups are tested) will be performed to determine the minimum number of animals needed to detect potential statistically significant differences. Based on previous results, we assume a variance of ~15-20% depending on the primary pain outcome measure. We will consider a power of 80-90% and a significance of at least 5%. In general, a minimal effect size of 30% is considered to be of biological relevance. We generally use 80% power as the standard, balancing the need to detect true effects while minimizing animal use. However, in critical confirmatory studies (go/no go) we increase power to 90%. This higher power reduces the risk of false negatives, ensuring more robust and reliable detection of potentially subtle or variable effects. If unexpected negative effects or adverse outcomes arise during the experiments, we will monitor these closely and perform additional analyses as needed to characterize their nature and impact. In such cases, we may adjust sample sizes or power calculations for follow-up studies to ensure sufficient sensitivity to detect and understand these effects. Interim analyses and consultation with statisticians and the Animal Welfare Body will guide any necessary modifications to minimize animal use while maintaining scientific rigor. In most experiments, the primary outcome will be pain assessment and in others, ex vivo analyses of cells/tissues will be the major basis of our method to calculate and minimize needed animals. Based on historical power calculations, we expect that the groups sizes will vary between 6-12 animals. The exact number for each study will be determined individually through power analyses to ensure the smallest number of animals is used without compromising scientific validity. All proposed group sizes and statistical plans will be reviewed and aligned with the Animal Welfare Body, which includes a qualified statistician, ensuring compliance with ethical standards and best practices.
For some research questions we will test 2 conditions in the same animal. For instance, in a model of inflammatory pain, intraplantar injection of an inflammatory substance in one paw and vehicle (e.g. saline) control in the other allows for testing both conditions in the same animal. Moreover, we will apply multiple procedures to measure different aspects of pain to reduce the number of animals needed. For example, tissues will be taken for ex vivo analysis where possible, so that a separate experiment (and extra animals) are not needed. In addition, if there are clear sex-differences in a specific model, we can choose to perform the experiments with one sex, to reduced variation and thereby reduce the number of animals needed.
For some research questions we will use genetically modified mice (e.g. fluorescent mitochondria). Our breeding policy includes a 2-weekly meeting with the involved scientist and regular discussions with the breeding coordinator of the GDL, to determine the number of animals needed to ensure optimal breeding strategies and avoid the breeding of unneeded animals. We will optimize our breeding strategies to produce minimal numbers of animals needed. For example, we will breed homozygous floxed mice with homozygous floxed mice that express one copy of a Cre/recombinase, so that all the progeny of the mating will either be experimental animals (i.e. genes will be deleted) or appropriate controls (ie floxed genes will be present but not deleted).

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
Click or tap here to enter text.	Mouse	Utrecht University, GDL or licensed suppliers (e.g Janvier)	Adults > 7 weeks	2980	Both	Both WT and GG	Various
	Rat	Utrecht University, GDL or Licensed	Adults >7 weeks	580	Both	WT	Various

	suppliers (e.g. Charles River)			

Provide justifications for these choices

Species	We will use mice and rats because these are the most commonly used species for pain research
Origin	Mice will be either bred at the GDL, University Utrecht or bought from licensed suppliers (e.g. Janvier). Rats are bought as well.
Life stages	We will mainly use adult animals. If experiments show that age-specific effects exist we might have to use a specific life stage.
Number	The general group size is expected to average 6-12 animals per group and is based on our experience with the group size calculations of our previous experiments (based on G-power calculations). For all objectives we estimate on average to require 4 experimental groups (e.g., variable 1: induction model vs SHAM/control, variable 2: strategy to interfere or vehicle). To test our objectives we expect to address our research questions by performing ~12 experiments/year. Therefore, we estimate to use a maximum of 576 ~ animals/year (=12 animals/group * 4 groups * 12 experiments), totaling to a maximum of 2880 mice for the 5-year period. Some models can currently only be performed in rats (groove model) and sometimes specific inhibitors/compounds are only available in rats. Therefore, we expect to address ~1 research questions/year that require ~1-2 experiments to address one research question. Therefore, we estimate to use a maximum of 72 ~ animals/year (=12 animals/group * 4 groups * 1 research questions/year * 2 experiments), totaling a maximum of 480 rats for the 5-year period. In addition to this number, we need a max of 20 mice/year (100 mice/5-years) and 20 rats/year (100 rats/5 years) to train new colleagues on the use of existing techniques. If possible, we will first perform cadaver training, but for some techniques live animals are needed under terminal anaesthesia (e.g. training intrathecal injections). However, for some practicing purposes (e.g. intraplantar injections), we will perform training without anaesthesia. We will use surplus animals for these purposes whenever possible. Total maximum number of experimental animals needed for this appendix is 2980 mice and 580 rats, totaling to 3560 animals.
Gender	We use both males and females. If experiments show that gender-specific effects exist, we may have to use one specific gender.
Genetic alterations	We will use Cre-loxP system to delete genes in specific cells or use global knockout mice that enable us to address questions about neuro-immune interactions in pain development. Specifically for the T2DM model, we will use mice which are transgenic for hIAPP and are obese due to an inactivating mutation in the Leptin gene (hIAPP/ObOb). As controls for these mice we need non-transgenic ObOb mice, mice transgenic for hIAPP but non-obese and WT mice, also on a C57Bl/6 background. ObOb and hIAPP ObOb mice have discomfort due to their obesity and hyperglycemia. For maintenance of these lines, we use heterozygous Ob x wildtype breeding pairs (the Ob mutation is recessive) and for generating homozygous Ob mice for experiments we use heterozygous Ob x heterozygous Ob breeding pairs. Since 25% of the latter offspring is homozygous Ob, these breedings will be performed under appendix 4 "breeding with (potential) discomfort"(see appendix 4)
Strain	In general, mainly C56Bl/6 mice will be used. They are the most commonly used mouse strain for pain studies and most knockout mice are on this background. Various mouse pain models have shown some translational value. However, for some specific questions we may need other strains because: 1) genetic modified mice have a specific background and backcrossing would cost many mice whilst not per se needed 2) the model/purpose of a study is easier to investigate in a specific strain. Currently we have a strain with discomfort (hIAPP/Ob or hIAPP only) mice. This will be bred under appendix 4 of this CCD. If we have new breedings, with unknown discomfort will also ask for an amendment to breed them under appendix 4.

	For our experiments in rats, we primarily plan to use WISTAR, as the models we aim to induce are most commonly established in this strain. However, if future studies in literature demonstrate that other strains better represent our models or improve their performance, we would be open to incorporating these into our research.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☒ Yes

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

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☐ No

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

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

We expect that the majority of animals experience pain, allodynia and hyperalgesia to some extent until the terminal phase of the experiment. This pain cannot be relieved using analgesics during the experiment as these pain parameters are the outcome measures of this project. However, pain induced by the operation itself, when actual pain measures are not required, will be dampened with appropriate analgesics. Additionally, soft white tissues will be added in the cage to reduce animal discomfort.

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

Anaesthesia will be used for intrathecal injections and intra-articular injection, for performance of groove model, and for injecting the tumor cells in the marrow cavity (CIBP model and for the neuropathic pain models). For the CIBP model (and for some neuropathic pain models) we will have to operate the animals, and we will use Xylocaine gel to reduce wound pain. Carprofen/Saline solution (5mg/kg) will be administered pre surgery to reduce wound pain. 24 hours post-surgery the animals will be monitored and if needed a second Carprofen/saline injection will be given. Additional soft tissues will be added to the cage to reduce animal discomfort.

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

In all models that include injection in the paw/knee, it can be expected that animals experience some oedema in the affected paw(s)/knee(s), but this should not hinder movement. For the CIBP model, it cannot be excluded that the bone tumor induces metastasis, for instance when the injection site is not completely filled with bone wax, in this case animals will be sacrificed. X-ray/Bioluminescence analysis will confirm if the tumor was successfully confined to the bone.
For T2DM model, (hIAPP)ObOb mice and ObOb mice are obese. From the age of 10 months onwards these animals can fall over and not stand up independently anymore, so mice will be killed before that age. hIAPP/ObOb mice develop hyperglycemia. Very rarely, after intrathecal injections paralysis may occur. In that case animals will be euthanized.

Explain why these effects may emerge.

These effects may emerge because of inflammation/damage to the paw/joints. Metastasis may emerge because of unsuccessful filling of the injected bone with bone wax. T2DM model: These effects are caused by the genetic predisposition of these mice.

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

When compounds are administered, the minimum effective dose will be used to minimise the chance of adverse effects and the most appropriate route of administration will be used. The animals will be observed in the period immediately after injection and if applicable, at least once daily afterwards. When any signs of extreme or unexpected reactions such as infection or swelling at the injection site, weight loss/gain or metastasis occur, the animal will be killed immediately. Wet food will be given, and additional tissues will be added in their home cages when appropriate. Hyperglycemia is the characteristic clinical feature of T2DM, therefore we cannot reduce this hyperglycemia with e.g. insulin administration, because that would make this animal model inappropriate.

E. 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 F

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Table 4: General HEP and model-specific HEPs

Category	HEP description
General HEPs	
	Animals exhibiting any unexpected harmful phenotypes, indicating severe discomfort, will be killed and advice will be taken from the Animal Welfare Body.
	Animals that show spontaneous pain behavior in the cage (frequent licking or shaking of the injected paws) will be euthanized (moderate).
	Animals that cannot walk normally (visible limping) will be weighed daily and if the weight loss is >15% within 2 days (moderate), they will be euthanized.
	Loss of more than 20% of their individual baseline weight.
	If any animal is seen to be having an extreme reaction (e.g. paw volume increases visibly in mild models and more than two-fold for moderate models) it will be killed humanely. Animals will also be monitored for altered social and grooming behaviour. The inflammation and associated pain should be restricted to the affected paw, so any signs of reduced general wellbeing will be taken as an extreme reaction, and the animal will be killed humanely.
	Immediate action should be taken if distention of the abdomen occurs, as well as abnormal vocalization when animals are touched or handled.
Model specific HEPs	
Bone pain models	Limb use: tumors (CIBP model) or knee damage (OA/RA models) may interfere with the free movement of the leg, thereby causing discomfort of animals and biasing the behavioural readout.
CIBP model	If there is no cell growth, metastasis (identified through radiography or bioluminescence), or Patella displacement.
In case of a surgery	Surgical complications; any signs of extreme or unexpected reactions such as infection or swelling at the surgery site
In case of intrathecal injections	Paralysis due to intrathecal injections

Indicate the likely incidence.

Based on our experience of previous CCDs, for all models this is very sporadically (<1 per 500), except for the CIBP model this is around 1:20 (e.g. due to luxation of the patella or metastasis). For specific research questions where we will use the CIBP model we will include this drop-out in our power calculation.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

All described inflammatory, neuropathic and bone pain models will give moderate discomfort. In these models we will apply a maximum of 3 different treatment strategies (procedures), to investigate whether we can attenuate the chronic pain (Table 5). Thus, all used procedures aim to reduce pain in these persistent pain models. In vehicle treated animals, pain probably persists, while in treated animals (with inhibitors, antibodies etc) the pain may attenuate. For all models we carefully monitor the HEP to **prevent** that mice experience severe discomfort. This is also in agreement with other international groups (e.g. University of Copenhagen, Denmark) using similar models as we use. Additionally, we have experience in all described models and we (scientists and caregivers) never had to report severe discomfort the last five years. However, according to the CCD, ' long-lasting' moderate discomfort should be classified as severe discomfort. Therefore, models in which mice experience moderate discomfort for more than four weeks will be classified as severe.

Expected discomfort:

Non-recovery = 3,3 %, since 100 mice (=3,35%) and 100 rats are used non-recovery for training purposes.
Mild= 16.7 % (497 mice)
Moderate = 80 % (2383 mice)

However, we expect that approximately 30% of the experiments will last more than four weeks, meaning that 715 mice (30% of 2383) will be classified as severe instead of moderate. Indicating that we finally will have approximately 1668 mice with moderate discomfort.

Of the chronic pain models, we expect that 25% will be used for the CIBP model (745 animals) and 15% for the T2DM model 447 animals). For CIBP, 1:20 will reach the humane endpoint, due to metastasis or luxation of the patella. However, we will detect this in an early stage (with radiograph/bioluminescence) so we can sacrifice the mice before the discomfort will become severe.

For the planned 480 rats for experiments, we will use the groove/TNT model which will give moderate discomfort. Sham-operated animals will have mild discomfort.
Thus, the expected discomfort in rats:
Mild = 20% (96 rats)
Severe = 80% (384 rats), since the duration of all experiments will exceed four weeks.

Table 5. Cumulative discomfort assessment for pain models and procedures. The light grey boxes indicate the selected "pain model" (mild or moderate), the mid-grey boxes are to indicate the selected procedure (one, two or three strategies), and dark-gray is the total expected cumulative discomfort.

Pain model		**Optional procedures**			**Pain measurement**	**Cumulative discomfort**
Vehicle-injected, sham operated **Mild**	Pain model **Moderate**	Strategies (X, Y, Z) are either i) application of compounds, ii) induction/deletion transgene or immune cell, iii) blood collection or, in case of bone pain models, microCT or in tumor models, bioluminescence. These procedures individually are classified as **mild** when only performed once, or **moderate** when performed multiple times. MicroCT we often do post-mortem, but in some case we will perform this in anaesthetised mice. In case a procedure has to be repeated multiple times we will employ max 2 optional procedures. **1st** **2nd** **3rd strategy**				
						Mild
						Mild
						Mild or Moderate
						Moderate
						Moderate
						Moderate
						Moderate
						Moderate (1 time procedure)
			Multiple times			**Moderate**
			Multiple times			**Moderate**

G. 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	As part of our studies, we will employ a range of in vitro techniques to investigate key metabolic and neuro-immune mechanisms underlying transient and chronic pain. These in vitro studies will enable us to get insights on specific cellular and molecular pathways, before transitioning to animal models. To investigate our research goals, we will employ both cell lines and primary cells. For the primary cells, animals will need to be sacrificed, as some key properties of cells can be lost when using cell lines. Additionally, we will use patient-derived material and induced pluripotent stem cells (IPSCs). For instance, with IPSC models we will generate sensory neurons, immune cells, and

	other relevant cell types (e.g. osteoclasts and osteoblasts) involved in pain regulation. Overall, these in vitro experiments yield valuable information to plan and execute our experiments in animals. While in vitro studies are important, they cannot provide a complete understanding of how pain is perceived, which is something we can only learn through animal research. Chronic pain is the result of complex interactions between signals generated by inflammation/damage, the sensory neurons at the site of the pain (like those in the paw), processing in the spinal cord, and the brain's response. In addition, a novel analgesic compound, with promising effects in vitro, must be tested in animals to measure its effects on pain development and resolution. Currently, it is impossible to recapitulate these systems in vitro. Thus, to better understand the mechanisms driving chronic pain, the interaction of these systems needs to be studied in living animals.
Reduction	First, we will optimise our breeding strategies to produce the minimal numbers of animals needed. Moreover, we will use males and female mice in our experiments, lowering the numbers of animals killed in our stock. We will reduce the animal numbers by not always repeating each experimental control group for each research question. For example, if we know that vehicle injections do not induce pain, we can leave this group out in some experimental set-ups. Thirdly, careful statistical analysis and experimental planning will be essential to reduce the numbers of animals needed. For instance, if the pain model allows injection in one paw and the animal can be used as its own control, this will be the preferred option, as it reduces the number of animals used. Fourth, we will apply multiple procedures to measure different aspects of pain and collect pain-related tissues from the same animals for ex vivo analysis. The collected tissues, the pain assessment and the ex vivo analysis, will contribute to a "pain database" that can be used for more experiments, reducing the number of animals needed. For instance, we may use RNA sequencing data of tissue sections collected in other experiments and add this information in this database to assess a new gene/protein or cell. Finally, if needed and possible, tissue from animals will be used to test, optimize and execute in vitro studies. For instance, bone marrow-derived cells may be collected from an animal and used to generate primary cultures for relevant analysis.
Refinement	We will design experiments in such a way that these experiments last as short as possible, because in general the degree of discomfort is time-dependent and age dependent. All planned models in this appendix have an estimated discomfort of moderate. Additionally, we will introduce home cage monitoring to be able to decrease the discomfort during behavioral testing. Furthermore, when the study allows, we will use analgesics to decrease discomfort (e.g. during/after operation). When oral administration is needed, we will explore the option to deliver treatment using micropipettes instead of gavage.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☐No

☒ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Some studies of chemical-induced pain (e.g CIBP) requires treatment with hazardous chemotherapy compounds. In these cases, treated animals will be caged in separate social cages, and their biological waste will be disposed accordingly to safety and disposal guidelines.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☒ No > Continue with question I.

☐ 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.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Not applicable

J. Location where the animals procedures are performed

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

☒ No > Continue with question K.

☐ 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.

Click or tap here to enter text.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

Click or tap here to enter text.

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

Animals will be used to collect tissue samples (e.g. DRG, sciatic nerves, bones, intestine) for further analysis. If we don't collect tissues, we will inform if the mice can be used for other experiments or training (ATEX).

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

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

Click or tap here to enter text.

☒ Yes > Will a method of killing be used for which specific requirements apply?

☐ No > Describe the method of killing.

Click or tap here to enter text.

☒ Yes > Describe the method of killing that will be used and provide justifications for this choice.

Animals will be mainly sacrificed using cervical dislocation or CO2 asphyxation. When animals need to be perfused, they will be sacrificed under sedation (followed by heart puncture) or with an overdose of euthesate. For rats, we will either perform heart puncture while the animal is under anesthesia or we will sacrifice the animal through an overdose of pentobarbital.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Click or tap here to enter text.

Centrale Commissie Dierproeven

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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMCU, Utrecht University
1.3	List the serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number: 2 Type of animal procedure: Transient pain models & measurements

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.

This appendix is similar to appendix 1, however we focus on transient pain models (with mild discomfort), to identify factors that are important for the resolution of pain as described in goal 1: Dissect neuro–immune interactions that drive resolution versus chronification of pain.

A general design of an animal experiment is illustrated in figure 1. For each experiment, animals can undergo different protocols/procedures.

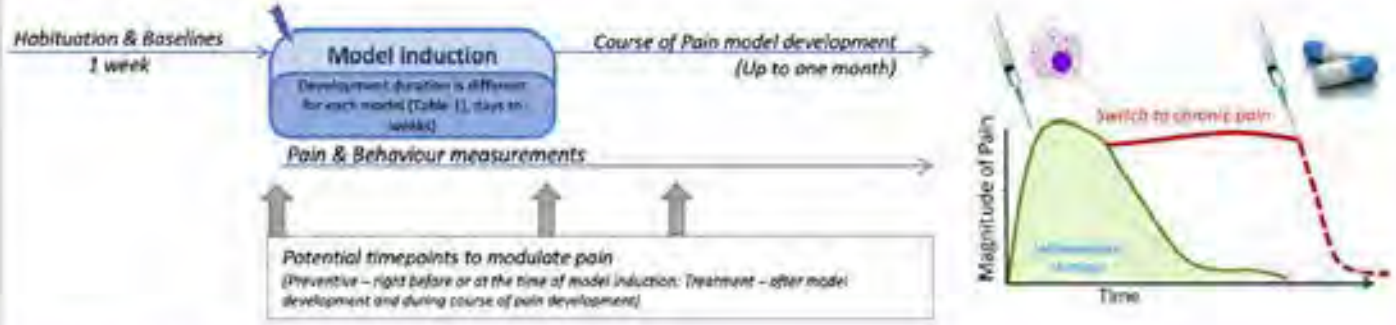


Figure 1: A general design of an animal experiment

Prior to the induction, pain-like behaviors and sensory thresholds are measured to obtain baseline values. Subsequently one model is selected from table 1 to induce transient pain. For example, we can inject a low dose of hIAPP to induce transient pain (mild discomfort) and collect pain-relevant tissues to determine pathways that are affected in immune and neuronal cells. We can also modulate pain at the start of the model, to investigate whether resolution of pain can be prevented. As example, before the start of a "model induction", we can deplete immune cells. From our previous work, we know that depletion of macrophages promote the switch from acute to chronic pain. If this transition happens, the discomfort also change from "mild" to "moderate". In this persistent pain phase we will test whether treatments can promote resolution of pain.

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

Each experiment has a general outline as shown in figure 2, with max. 3 procedures to modify genes, signalling cascades, or cells and different moments that we cull mice for e.g. ex vivo analysis. The general outline is detailed below, in which we describe 3 experimental options.

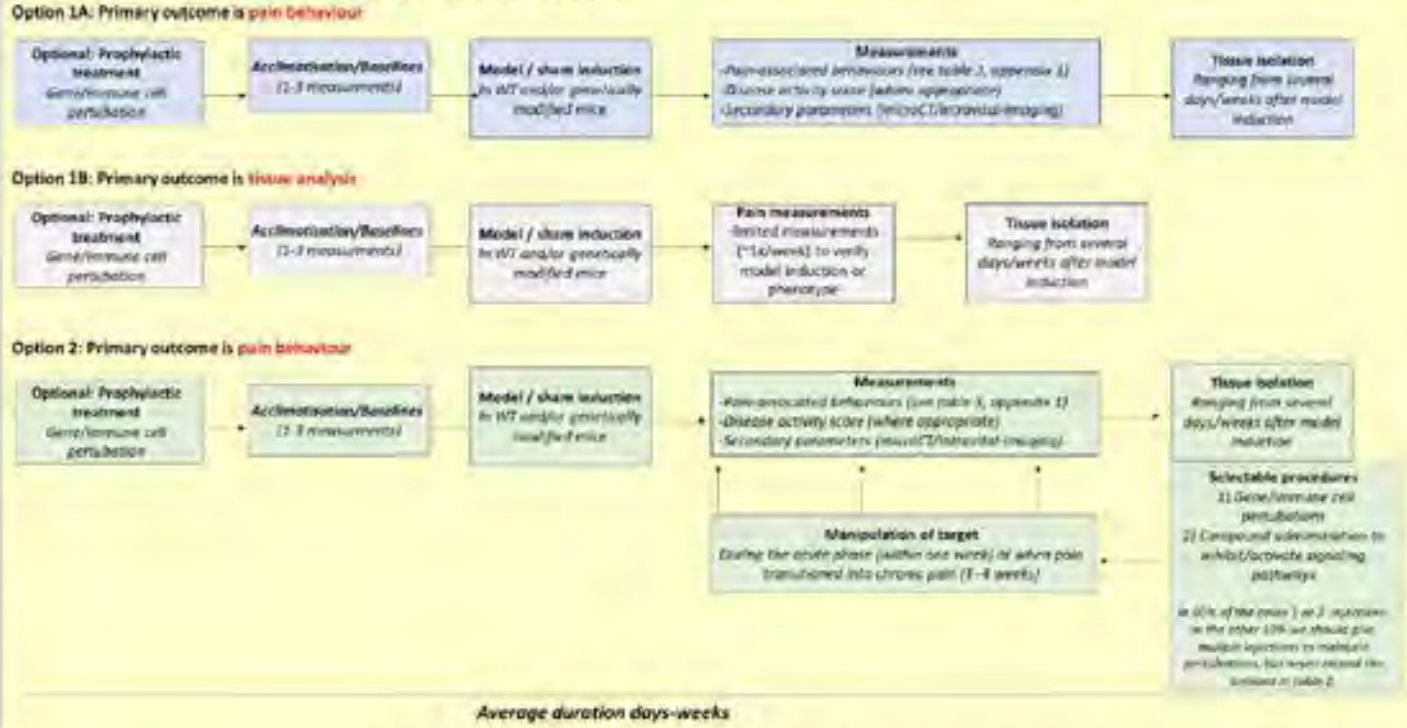


Figure 2: schematic overview of a design of experiments and optional procedures that can be used for each model. The procedure for model induction is shortly (e.g. intrathecal injection is max 3 minutes), but the consequence is that pain symptoms may develop that persist over a longer time period, ranging from several days to weeks. However the procedure could also induce pain resolution and thereby stop the pain.

We will use transient inflammatory and neuropathic pain models because these types of pain have next to some central common/shared mechanisms, also distinct mechanisms that contribute to the pain. Therefore, it will be important to investigate our proposed mechanisms in one model and sometimes also in 1-2 other models per pain type to be able to tell whether that specific mechanism is a pain type specific or represent a common mechanism. Of note, bone pain does not have a transient equivalent. As one type of pain cannot be represented by a single model, we need to have the ability to choose a model depending on the current literature and discussions with our scientific collaborators, to ensure the least amount of models/mice used, whilst increasing our scientific impact and generate an optimal dataset necessary to develop novel pain strategies. Finally, all these models represent one type of animal experiment with variations in the induction, intervention and specific selection of pain measurement. See figure below.

The precise choice for the model(s) to use will depend on literature and our in vitro research to make a rational choice for prioritizing the best model for each specific research question. After investigating a phenomenon/mechanism we may want to test whether such a mechanism is specific for this model or whether it also applies to other models and other types of pain. For example, if we find a protein linked to pain resolution in humans (based on proteomics and clinical data), we will target this protein (by using inhibitors, antibodies, genetically modified mice), to investigate whether pain resolution fails. If our behavioral measurement shows that this protein is important to regulate pain resolution, we will verify these findings in another model/type of pain (see Figure 3, project proposal).

*Table 1: Different transient pain models that could be used in mice. The two **bold** models are the preferred starting models.*

INFLAMMATORY PAIN

Agent	Dose	Route	Frequency	Max Duration	Anesthesia	Analgesics	Severity
Inflammatory soup	Carrageenan (1% (w/v), 5 µl) or CFA (2.5 µl diluted 1:1 in saline)	Intraplantar	1x	7 days	No	No	Mild
Single inflammatory mediators (eg PGE2, IL1b, CCL2, NGF)	1 pg-1µg	Intraplantar	1x	7 days	No	No	Mild

NEUROPATHIC PAIN MODELS

Chemical induced pain models							
Chemotherapy-induced	E.g. Paxlitaxel (2 mg/kg)	Intraperitoneal	2x	14 days	No	No	Mild
Neurotoxic (amyloid-induced)	1-10 fg	Intraplantar	1x	8 days	No	No	Mild
Mechanical induced pain models							
Acute Postsurgical pain	Operation	Plantar incision hindpaw, followed closure using silk sulfunes	1x	7 days	Yes	No	Mild

<u>Explanation of the approached models</u>

Based on our extensive experience with the models described below, we can reliably classify all of them as involving mild discomfort in accordance with the EU Directive 2010/63/EU and Dutch legislation. This classification is supported by previous use, observed animal responses, and established literature. The choice of which model to use is mainly dependent on the research question and on existing knowledge/data, which will be further explained below by each specific model.

Inflammatory pain models

Low dose carrageenan/CFA

CFA and carrageenan induces an "inflammatory soup of mediators" and can be injected either in 1 (unilateral) or 2 (bilateral) hind paws, depending on the experiment setup that requires the fewest animals. For example, if a compound is being tested locally by intraplantar injection, an inflammation in both paws will enable to test vehicle and drug in the same animal. Moreover, in some cases for ex vivo analysis of tissue (e.g. sensory neurons innervating the affected area), the amount of obtained tissue (unilateral) is not sufficient, whilst when the model is bilateral, we will obtain sufficient material from 1 mouse. If the setup allows injection in one paw without increasing the required number of animals, this will be the preferred option as it reduces animal discomfort. Low dose of carrageenan will be our first choice model if we want to investigate if an identified neuro-immune factor (e.g [redacted] solution of pain. This model we already used often in our researc[redacted] As example, [redacted] pletion of monocytes/macrophages in this model promoted [redacted] chronic pai[redacted] ndicating that monocytes/macrophages are important for the resolution of inflammatory pain (by [redacted] ondria). Low dose of CFA is often used as verification model.

Neuropathic pain models

Neurotoxic (amyloid-induced)

Amyloid deposits of human islet amyloid polypeptide (hIAPP) and associated tissue dysfunction are found not only in the pancreatic islets, where hIAPP is produced by β-cells, but also in the heart, kidneys, and brain of T2DM patients (Gong et al., 2007; Liu et al., 2016; Jack[redacted] ecently we observed that a low dose of hIAPP in hindpaws of mice induces transient hypersensitivit[redacted] llowing us to investigate which resolution mechanisms are important to resolve amyloid-induced ne[redacted] ng diabetic-neuropathy this will [redacted] el. When we investigate novel therapeutic options to target hIAPP, we will mainly use this mode[redacted]

Chemotherapy-induced peripheral neuropathy (CIPN)

Chemotherapy-induced pain is developed following the administration of chemotherapeutic compounds such as paclitaxel or oxaliplatin via intraperitoneal administration. Low dose of these compounds will induce transient neuropathy, allowing us to investigate which resolution mechanisms are important to resolve chemotherapy-induced neuropathy. We know that chemotherapy can affect immune cells. This will mainly be a verification model

Chronic postsurgical pain (CPSP)

The hind paw incisional model is a reliable and feasible approach to investigating the mechanisms underlying neuropathic pain and healing. Briefly, it is described as a 5-mm long midline incision made through the plantar skin fascia starting 2 mm from the heel and extending towards the toes on the left hind paw, followed by the skin incision closure using silk sutures. (Cowie et al, BioProtoc 2019). This model is recently used as verification model to test whether monocyte/macrophage depletion also prolongs CPSP. However, we could also use CPSP as starting model, if we have evidence from literature that this model is the best to start with. For example, increased elastase levels (produced by T-cells and neutrophils) are found after post-surgical injury and may regulate inflammation and neuropathic pain (PMID: 7721460; 25951529). So for this research we will start with CPSP model to test whether knockout mice for elastase have increased and prolonged post-operative pain.

To give an idea about the use of the transient models. Last CCD we used approximately in 70% of the work protocols a low dose of carrageenan model, 10% the CFA model, 10% the hIAPP model and 10% the CPSP/CIPN models. However in this CCD this distribution can be a bit different. For example, we are currently investigating whether an antibody targeting hIAPP can prevent pain development. So we expect to use more the hIAPP model upcoming 5 years.

Administration compounds/substances

To investigate and understand the molecular mechanisms of pain, different compounds or control substances (alone or in combination, continuously or intermittently) will be administered by one or multiple routes. A schematic description of the drug administration routes (including information such as maximum frequency, maximum number of administration within one experiment and maximum volumes) are reported in Table 2.

Table 2: Schematic description of drug administration routes, maximum volume and frequency following the international guidelines. In practice we never come to the maximal frequencies (so uses less).

		Mouse		
Route	**Anaesthesia**	**Max Frequency**	**Max total administration**	**Max volumes**
Subcutaneous	No	2 x day	20 injections	10 ml/kg
Intraperitoneal	No	2 x day	30 injections	20 ml/kg
Intravenous	No	1 day	Max 7 injections	5 ml/kg
Intrathecal	Yes	1 x day	8 injections	7.5 µl
Intra-articular	Yes	1 x day	8 injections	50 µl
Intraplantar	No	1 x day	5 injections	25 µl
Oral (gavage or micropipette)	No	2 x day	Up to 28 days	10 ml/kg

The compound and concentration will be determined based on in vitro evidence for a role in the regulation of cells, evidence in literature including information on dosage, selectivity, or mechanism of action. Care will be taken to avoid potential known side-effects at certain concentrations. The route and number of applications will be determined based on inducing the least discomfort but sufficient to block or stimulate a specific pathway at a specific certain period of time.

Behavioral assessments

For all models we will do behavioral assessment and we will use 1 or several assessments, depending on the research question with a **maximum of 4 behaviour tests** per day/per animal (table 3)). For example, we will test mechanical sensitivity (Von-Frey), thermal sensitivity (Hargreaves), locomotor function (rotarod) and cognition (puzzle box). This combination of tests will allow us to address different behaviours. In this example of multiple assessments, each test will be performed only once in a day. The choice of tests depends on the animal model that is being used and the pathology it represents. The animals will be acclimatized to the testing environment to minimize stress and fear during the measurements. See table 3 () and associated text to explain the models.

*Table 3: Possible behavioral assessments. Predominantly **two** "black assessments" are used in our experiments. They can be extended with one or two "black or grey assessments", depending on research question (e.g. chemotherapy can induces sensitivity to cold, so cold plate test can be added). *max 7 sessions in a 7 day period, can be at the start of the induction model or during the persistent phase if we just gave a procedure (e.g. to monitor if a compound reduces pain)*

	Method	Stimulus	Maximum stimulus duration	Maximum Frequency*	Species
Evoked-hypersensitivity	Von Frey	Calibrated series of von-Frey hairs ranging from 0.02 to 4g	5 seconds per hair	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
	Hargreaves	Focused light beam (infrared) using a Hargreaves apparatus	Max 20 seconds per paw	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
	[illegible]	[illegible]	15 min	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
Non-Evoked-hypersensitivity	Dynamic weight bearing	No stimulus	5 min	Max 7 sessions in a 7-day period. During the persistent phase we only measure max 1 a 2x per week	Mouse
	Place-conditioning test	Administration of a pain relieving compounds	2x 30 minutes	Max 5 days	Mouse
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
Activity & cognition	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	Homecage monitoring Videotaping	No stimulus	24/7	24/7	Mouse
	Open field, Videotaping	No stimulus	1 hour	[illegible]	Mouse
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

In table 3 we separated the different methods to measure evoked-hypersensitivity, non-evoked hypersensitivity (spontaneous pain), activity and cognition. We shortly explain each method:

Evoked-hypersensitivity

Von Frey
Mechanical threshold will be measured using von Frey monofilaments (Chaplan SR, et al., Quantitative assessment of tactile allodynia in the rat paw. J Neurosci Methods. 1994). In brief, animals will be placed on a wired grid base in Perspex boxes, through which the von Frey hairs will be applied (bending force range from 0.02 to 2 gr, starting with 0.4 gr) on the affected paw. The hair force will increase or decrease depending on the response. Clear paw withdrawal, shaking or licking will be considered a positive response. Ambulation will be considered as an ambiguous response, and the stimulus will be repeated.

Hargreaves:
For the Hargreaves test animals will be placed on a warm glass plate (32 degrees) in Perspex boxes (Hargreaves K, et al., J. A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. Pain. 1988). A light beam will be pointed at the hind paw to induce heat in the paw and latency time until the animals react will be recorded. The maximum stimulus duration is 20s and the machine will automatically turn off the light.

Thermal sensitivity:
Animals are placed on a metal plate where temperatures range from 0-65°C (Yarchi J, et al., Differentiating thermal allodynia and hyperalgesia using dynamic hot and cold plate in rodents. J Pain. 2009). Latency to first response is recorded (clear paw withdrawal, shaking or licking will consider as positive responses).

Non-evoked hypersensitivity
Dynamic weight bearing:
The automated DWB device consists of a Plexiglas enclosure (11×11 cm for mice, 22x22 cm for rats), a floor sensor composed of 44 × 44 captors detecting pressure variation and a camera placed above the enclosure in order to validate the animal position (Robinson I et al., Use of dynamic weight bearing as a novel end-point for the assessment of Freund's Complete Adjuvant induced hypersensitivity in mice. Neurosci Lett. 2012.). The animals are able to move freely in the enclosure during a total time of 10 min, starting with a habituation phase of 5 min followed by a recording phase of 5 min in which the weight distribution and the video will be recorded. The data will be analysed offline.

Conditioned Place Preference (CPP):
In the CPP, a 3-chamber (A,B, and C) box is used, with the B chamber being a smaller chamber connecting A and C, separated from the neighboring chambers by a divider with an entrance (He Y, et al., Negative reinforcement reveals non-evoked ongoing pain in mice with tissue or nerve injury. J Pain. 2012). The A and C chambers have various patterned walls and floors that are texturally different. On day 1 (preconditioning), animals will be allowed to move freely for 30 minutes, and the time spent in each chamber will be recorded. On days 2–4, the animals will be conditioned with daily intraperitoneal injections of vehicle or gabapentin. In the morning, the animal will be placed for 30 minutes in 1 closed chamber (black room), 1 minute after receiving the vehicle. In the afternoon (3 hours later), the animal will be placed for 30 minutes in the other chamber (white room), 1 minute after receiving gabapentin treatment. On day 5, after conditioning, the animals will be placed in the connecting chamber (chamber B) and allowed free access for 30 minutes to chamber A or C. The time spent in the chambers will be recorded.

Grimacing Videotaping: Animals will be videotaped to analyze facial expression over time that are linked to spontaneous pain, such as orbital tightening, nose/cheek bulge, ear position, etc (Deuis JR, et al., Methods Used to Evaluate Pain Behaviors in Rodents. Front Mol Neurosci. 2017).

Vocalisation:
Systems will be used to measure ultrasounds (e.g. sonotrack system) in a special environment that is closed for outsides noises (Williams WO. et al., Ultrasonic sound as an indicator of acute pain in laboratory mice. J Am Assoc Lab Anim Sci. 2008).

Burrowing assay
A long tube (200 mm length x72 mm diameter, raised 30 mm from the ground in the frontal sealed at one end is filled with a substrate (cage bedding) and placed in a standard transparent plastic cage without bedding (Deacon RM. Burrowing in rodents: a sensitive method for detecting behavioral dysfunction. Nat Protoc. 2006). Each mouse is placed individually in the cage with the described setup. After 2 hours, the amount of material displaced is weighed and recorded. The reduction in the amount of substrate voluntarily burrowed can be used as a surrogate marker of pain-like behaviour.

Grid climbing test
Each animal is placed on a standard wire lid (I Falk S, et al., Grid-climbing behaviour as a pain measure for cancer-induced bone pain and neuropathic pain. In Vivo. 2017). The lid is turned upside down with the animal now hanging from underneath for 2.5 min while being filmed from above. Each film is subsequently analysed in slow-motion and the time spent using the affected leg is recorded manually.

In addition to the pain assessments, we sometimes perform behavioural tests to assess secondary outcome parameters. For instance, rotarod or open field may be employed to assess general motor performance after treatment/experimental strategy to exclude impaired motor behaviour which could affect the behavioural pain assays. Or for some models we want to measure cognitive functions, since we know that these can be impaired after chemotherapy or in patients with post-infectious syndromes (e.g., post-COVID).

Activity & cognition
Puzzle Box Test: Mice are individually placed in an open perspex chamber with a tunnel connecting to a closed/dark chamber (Abdallah NM. et al., The puzzle box as a simple and efficient behavioral test for exploring impairments of general cognition and executive functions in mouse models of schizophrenia. Exp Neurol. 2011). Mice rapidly prefer this dark/goal chamber. The time to enter the goal chamber is measured at 3 levels of complexity (across 3days/9trials): tunnel left open, tunnel covered by bedding material and tunnel covered by a plug. Each mouse performs 3trials per day during 3 consecutive days. Each trial has a maximum of 3 min (training and burrowing) or 6 min (plug), even if the mouse fails to solve the task. All sessions are recorded for later analysis.

Home-cage monitoring:
We purchased a home-cage monitoring system (Noldus). This system allows us to measure 2 mice per cage 24/7 for behavior. The advantage is that we can measure behavior for a long time (instead of 30 min) and also during the night, the time when they often show their natural behavior better. We also need to remove the animals less from their home-cage, which gives less stress/anxiety and should bring less variation in the data (less examiner influences). With this, we don't need the rotarod and open field setup. We only need to do Von Frey followed by monitoring in the home-cage. This also allows us to replace the puzzle box with the cognitive wall in the home cage. For this cognitive test, the mice do need to be individually housed for 8-9 hours. (Cassidy BR, et al., Progressive cognitive impairment after recovery from neuroinvasive and non-neuroinvasive Listeria monocytogenes infection. J Infect Dis. 2025)

Open Field:
Animals will be placed in a cage without bedding. Activity will be tracked by video camera above the cage for 30 min (Kraeuter AK & Guest PC, Sarnyai Z. The Open Field Test for Measuring Locomotor Activity and Anxiety-Like Behavior. Methods Mol Biol. 2019).

Rotarod: Animals will be placed on the rotarod (Vandeputte, C., et al. Automated quantitative gait analysis in animal models of movement disorders. *BMC Neurosci* 2010). We will apply two different measures. 1: Speed of roterod will increase from 4 rpm to 40rpm in 300 sec. Latency until the animal falls of or does a "passive rotation" will be recorded. In total 3 trial will be performed with 15 min at least between trials. 2: 10 rpm for 15 min. Time until animals stop walking will be recorded

Recognition memory:
This test consists in 3 phases: Habituation to arena: animals are placed in the empty arena and allowed to explore for 10 min. The next day there is a training or familiar session: introduction to the arena with 2 familiar objects, return to cage, followed by the replacement of one object with a novel object for the testing session (maximum of 10 min each session). If considering place recognition memory, there is an intermediat session where one of the familiar objects is moved to a new location first and then replaced in this location (Vogel-Ciernia A. & Wood MA. Examining object location and object recognition memory in mice. Curr Protoc Neurosci. 2014).

In addition to the behavioral assessments, we sometimes perform tests to assess secondary outcome parameters. For example, to verify good T2DM induction, we will characterize glucose homeostasis/metabolism (basal blood glucose, HbA1c and insulin levels, ITT (insulin tolerance test) and GTT (glucose tolerance test), degree of inflammation (body temperature and blood cytokine levels), degree of amyloidosis, blood levels of amyloid proteins and bodyweight. In the models where we inject compounds in the paw, we will measure the degree of inflammation by measuring paw or knee thickness after intraplantar injections. If required to measure compounds in blood, blood will be collected from a superficial vessel (totalling < 10 % of circulating volume) at different time points during the experiment. Furthermore, in bone pain models, we will employ microCT imaging (Int J Mol Sci. 2022:23 (7): 3725) to monitor disease progression in surgical and chemically-induced models (for instance in the Groove model and the MIA model) and to evaluate the effectiveness of potential treatments not only in alleviating pain but also in improving joint damage, by comparing microCT images taken before and after treatment. In addition, for some of the research questions, we perform intravital imaging of the spinal cord, DRG and nerves in anesthetized animals. We image genetically labelled fluorescent adducts for organelle-specific (e.g. mitotracker) or metabolic-specific dyes (e.g mitosox for mitochondrial ROS production) while animals are under anesthesia. Laminectomy will be used to visualize the DRGs, nerves, spinal cord. In vivo imaging will allow us to study dynamic processes that are missed in static analysis.

At the end of all the experiments blood, all relevant tissues and/or organs will be collected for *ex-vivo* analysis to reduce the number of animals required. There is a possibility that new ways for pain assessment are being published or developed in the coming 5 years. We would like to be able to include these models if they have shown to produce more reliable results or are less invasive for the animals.

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

For each experiment, power calculation (with Bonferroni correction if multiple groups are tested) will be performed to determine the minimum number of animals needed to detect potential statistically significant differences. Based on previous results, we assume a variance of ~15-20% depending on the primary pain outcome measure. We will consider a power of 80-90% and a significance of at least 5%. In general, a minimal effect size of 30% is considered to be of biological relevance. We generally use 80% power as the standard, balancing the need to detect true effects while minimizing animal use. However, in critical confirmatory studies we increase power to 90%. This higher power reduces the risk of false negatives, ensuring more robust and reliable detection of potentially subtle or variable effects. If unexpected negative effects or adverse outcomes arise during the experiments, we will monitor these closely and perform additional analyses as needed to characterize their nature and impact. In such cases, we may adjust sample sizes or power calculations for follow-up studies to ensure sufficient sensitivity to detect and understand these effects. Interim analyses and consultation with statisticians and the Animal Welfare Body will guide any necessary modifications to minimize animal use while maintaining scientific rigor. In most experiments, the primary outcome will be pain assessment and in others ex vivo analyses of cells/tissues will be the major basis of our method to calculate and minimize needed animals. Based on historical power calculations, we expect that the groups sizes will vary between 6-12 animals. The exact number for each study will be determined individually through power analyses to ensure the smallest number of animals is used without compromising scientific validity. All proposed group sizes and statistical plans will be reviewed and aligned with the Animal Welfare Body, which includes a qualified statistician, ensuring compliance with ethical standards and best practices.
For some research questions we will test 2 conditions in the same animal. For instance, in a model of inflammatory pain, intraplantar injection of an inflammatory substance in one paw and vehicle (e.g. saline) control in the other allows for testing both conditions in the same animal. Moreover, we will apply multiple procedures to measure different aspects of pain to reduce the number of animals needed. For example, tissues will be taken for ex vivo analysis where possible, so that a separate experiment (and extra animals) are not needed. In addition, if there are clear sex-differences in a specific model, we can choose to perform the experiments with one sex, to reduced variation and thereby reduce the number of animals needed.
For some research questions we will use genetically modified mice (e.g. fluorescent mitochondria). Our breeding policy includes a 2-weekly meeting with the involved scientist and regular discussions with the breeding coordinator of the GDL, to determine the number of animals needed to ensure optimal breeding strategies and avoid the breeding of unneeded animals. We will optimize our breeding strategies to produce minimal numbers of animals needed. For example, we will breed homozygous floxed mice with homozygous floxed mice that express one copy of a Cre/recombinase, so that all the progeny of the mating will either be experimental animals (i.e. genes will be deleted) or appropriate controls (ie floxed genes will be present but not deleted).

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
Click or tap here to enter text.	Mouse	Utrecht University, GDL or licensed suppliers (e.g Janvier)	Adults > 7 weeks	1440	Both	Both WT and GG	Various

Provide justifications for these choices

Species	We will use mice because these are the most commonly used species for pain research
Origin	Mice will be either bred at the GDL, University Utrecht or bought from licensed suppliers (e.g. Janvier).
Life stages	We will mainly use adult animals. If experiments show that age-specific effects exist we may have to use one specific life stage.
Number	The general group size is expected to average 6-12 animals per group and is based on our experience with the group size calculations of our previous experiments (based on G-power calculations). For all objective we estimate on average to require 4 experimental groups (e.g., variable 1: induction model vs SHAM/control, variable 2: strategy to interfere or vehicle). For testing our objectives we expect to address our research questions by performing ~6 experiments/year/objective. Therefore, we estimate to use a maximum of 288 ~ animals/year (=12 animals/group * 4 groups * 6 experiments), totaling to a maximum of 1440 mice for the 5-year period.
Gender	We use both males and females. If experiments show that gender-specific effects exist, we may have to use one specific gender.
Genetic alterations	We will use Cre-loxP system to delete genes in specific cells or use global knockout mice that enable us to address questions neuro-immune interactions in pain development. Breedings will be performed under appendix 4 "breeding with (potential) discomfort"(see appendix 4)
Strain	In general, mainly C56Bl/6 mice will be used. They are the most commonly used mouse strain for pain studies and most knockout mice are on this background. Various mouse pain models have shown some translational value. However, for some specific questions we may need other strains because: 1) genetic modified mice have a specific background and backcrossing would cost many mice whilst not per se needed 2) the model/purpose of a study is easier to investigate in a specific strain.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☒ Yes

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

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☐ No

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

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

We expect that the majority of animals experience mild pain, allodynia and hyperalgesia for a limited period of time. This pain cannot be relieved using analgesics during the experiment as these pain parameters are the outcome measures of this project.

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

Anaesthesia will be used for intrathecal injections.

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

In all models, which include injection in the paw, it can be expected that animals experience some oedema in the affected paw(s), but this should not hinder movement. In some rare case, intrathecal injection may induce paralysis. In that case animals will be euthanized.

Explain why these effects may emerge.

These effects may emerge because of inflammation/damage to the paw.

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

When compounds are administered the minimum effective dose will be used to minimise the chance of adverse effects and the most appropriate route of administration will be used. The animals will be observed in the period immediately after injection and if applicable at least once daily afterwards. When any signs of extreme or unexpected reactions such as infection or swelling at the injection site, weight loss/gain, the animal will be killed immediately. Wet food will be given, and additional tissues will be added in their home cages when appropriate.

E. 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 F

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Table 4: General HEP and model-specific HEPs

Category	**HEP description**
General HEPs	
	Animals exhibiting any unexpected harmful phenotypes, indicating severe discomfort, will be killed and advice will be taken from the Animal Welfare Body.
	Animals that show spontaneous pain behavior in the cage (frequent licking or shaking of the injected paws) will be euthanized (moderate).
	Animals that cannot walk normally (visible limping) will be weighed daily and if the weight loss is >15% within 2 days (moderate), they will be euthanized.
	Loss of more than 20% of their individual baseline weight.
	If any animal is seen to be having an extreme reaction (e.g. paw volume increases visibly in mild models and more than two-fold for moderate models) it will be killed humanely. Animals will also be monitored for altered social and grooming behaviour. The inflammation and associated pain should be restricted to the affected paw, so any signs of reduced general wellbeing will be taken as an extreme reaction, and the animal will be killed humanely.
	Immediate action should be taken if distention of the abdomen occurs, as well as abnormal vocalization when animals are touched or handled.
Model specific HEPs	
In case of a surgery	Surgical complications; any signs of extreme or unexpected reactions such as infection or swelling at the surgery site
In case of intrathecal injections	Paralysis due to intrathecal injections

Indicate the likely incidence.

Based on our experience of previous CCDs, for all models this is very sporadically (<1 per 500)

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

All described models will give mild discomfort. In these models we will apply a maximum of 3 different treatment strategies, to investigate whether we can prevent/attenuate the chronic pain. Depending on the frequency and route (e.g. intrathecal) of administration the cumulative discomfort can be rated as moderate at maximum (Table 4). For example, if we will use a transient pain model (mild) and modify gene expression levels by giving an intrathecal injection (in which the mice go under anaesthesia) with e.g. antisense oligonucleotides, the total discomfort is mild. However, if we combine a transient pain model and change the procedure to multiple intrathecal injections (moderate),

instead of one intrathecal injection (mild), the total discomfort will be moderate. The possibility exists that a strategy promotes the chronification of a transient pain model, for example, by inhibiting compounds/cells which normally are important to resolve pain. In this case the cumulative discomfort is estimated to be moderate at maximum. For all models we carefully monitor the HEP to **prevent** that mice experience severe discomfort. This is also in agreement with other international groups (e.g. University of Copenhagen, Denmark) using similar models as we use. Additionally, we have experience in all described models and we (scientists and caregivers) never had to report severe discomfort the last five years.

Optional procedures could increase the severity of the models from mild to moderate. For example if a procedure (e.g., knockdown of a specific gene) prolongs the hypersensitivity of mice in that case the animals will have a moderate cumulative discomfort.

Expected discomfort:
Mild = 50%
Moderate = 50%

Table 5: Cumulative discomfort assessment for pain models and procedures. The light grey boxes indicate the selected "pain model" (mild or moderate), the mid-grey boxes are to indicate the selected procedure (one, two or three strategies), and dark-gray is the total expected cumulative discomfort.

Pain model	Optional procedures			Pain measurement	Cumulative discomfort
Vehicle-injected, sham operated or transient pain model Mild	Strategies (X, Y, Z) are either i) application of compounds, ii) induction/deletion transgene or immune cell, iii) blood collection In case a procedure has to be repeated multiple times we will employ max 2 optional procedures. 1st	2nd	3rd strategy		
					Mild
					Mild
					Mild or Moderate
					Moderate
		Multiple times			Moderate

G. 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	As part of our studies, we will employ a range of in vitro techniques to investigate key metabolic and neuro-immune mechanisms underlying transient and chronic pain. These in vitro studies will enable us to get insights on specific cellular and molecular pathways, before transitioning to animal models. To investigate our research goals, we will employ both cell lines and primary cells. For the primary cells, animals will need to be sacrificed, as some key properties of cells can be lost when using cell lines. Additionally, we will use patient-derived material and induced pluripotent stem cells (iPSCs). For instance, with iPSC models we will generate sensory neurons, immune cells, and other relevant cell types (e.g. osteoclasts and osteoblasts) involved in pain regulation. Overall, these in vitro experiments yield valuable information to plan and

	execute our experiments in animals. While in vitro studies are important, they can't provide a complete understanding of how pain is perceived, that's something we can only learn through animal research. Chronic pain is the result of complex interactions between signals generated by inflammation/damage, the sensory neurons at the site of the pain (like those in the paw), processing in the spinal cord, and the brain's response. In addition, a novel analgesic compound, with promising effects in vitro, must be tested in animals to measure its effects on pain development and resolution. Currently, it is impossible to recapitulate these systems in vitro. Thus, to better understand the mechanisms driving chronic pain, the interaction of these systems needs to be studied in living animals.
Reduction	First, we will optimise our breeding strategies to produce the minimal numbers of animals needed. Moreover, we will use males and female mice in our experiments, lowering the numbers of animals killed in our stock. We will reduce the animal numbers by not always repeating each experimental control group for each research question. For example, if we know that vehicle injections do not induce pain, we can leave this group out in some experimental set-ups. Thirdly, careful statistical analysis and experimental planning will be essential to reduce the numbers of animals needed. For instance, if the pain model allows injection in one paw and the animal can be used as its own control, this will be the preferred option, as it reduces the number of animals used. Fourth, we will apply multiple procedures to measure different aspects of pain and collect pain-related tissues from the same animals for ex vivo analysis. The collected tissues, the pain assessment and the ex vivo analysis, will contribute to a "pain database" that can be used for more experiments, reducing the number of animals needed. For instance, we may use RNA Sequencing data of tissue sections collected in other experiments and add this information in this database to assess a new gene/protein or cell. Finally, if needed and possible, tissue from animals will be used to test, optimize and execute in vitro studies. For instance, bone marrow-derived cells may be collected from an animal and used to generate primary cultures for relevant analysis.
Refinement	We will design experiments in such a way that these experiments last as short as possible, because in general the degree of discomfort is time-dependent and age dependent. Additionally, we will introduce home cage monitoring to be able to decrease the discomfort during behavioral testing. Furthermore, when the study allows, we will use analgesics to decrease discomfort (e.g. during/after operation). When oral administration is needed, we will explore the option to deliver treatment using micropipettes instead of gavage. 1

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☐No

☒ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Some studies of chemical-induced pain require treatment with hazardous chemotherapy compounds. In these cases, treated animals will be caged in separate social cages, and their biological waste will be disposed accordingly to safety and disposal guidelines.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☒ No > Continue with question I.

☐ 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.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Not applicable

J. Location where the animals procedures are performed

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

☒ No > Continue with question K.

☐ 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.

Click or tap here to enter text.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

Click or tap here to enter text.

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

Animals will be used to collect tissue samples (e.g. DRG, sciatic nerves, bones, intestine) for further analysis. If we don't collect tissues, we will inform if the mice can be used for other experiments or training (ATEX).

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

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

Click or tap here to enter text.

☒ Yes > Will a method of killing be used for which specific requirements apply?

☐ No > Describe the method of killing.

Click or tap here to enter text.

☒ Yes > Describe the method of killing that will be used and provide justifications for this choice.

Animals will be mainly sacrificed using cervical dislocation or CO2 asphyxation. When animals need to be perfused, they will be sacrificed under sedation (followed by heart puncture) or with an overdose of euthesate.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Click or tap here to enter text.

Centrale Commissie Dierproeven

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 on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500	
1.2	Provide the name of the licenced establishment.	UMCU, Utrecht University	
1.3	List the serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number	Type of animal procedure
		3	Obtaining donor cells & cells for ex vivo analysis

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.

Over the years we and other research groups have discovered that pain resolution is not simply a passive process driven by the dissipation of inflammatory and tissue-damage mediators, but rather an active process involving the immune system. For example, we recently observed that adoptive transfer of anti-inflammatory macrophage[redacted] solution of pain, while macrophages with reduced mitochondrial potential failed to do [redacted] We used ex vivo cultures of mouse DRG neurons to test how macrophages and neurons [redacted] hat macrophages can donate mitochondria to neurons in an in vitro and in vivo setting [redacted]

From recent work by us and others we know that other immune cells, such as T-cells and neutrophils, infiltrate into pain-relevant areas, such as the DRG, in both chronic and transient pain rodent models. Depending on their phenotype these immune cells can promote, maintain or resolve pain. We still lack in understanding of what the role of these immune cells in DRG is and whether they communicate with neurons or other cell types within the DRG, like satellite glia cells.

We aim to isolate;

1) tissues, like DRGs, to culture pain-relevant cells (e.g. neurons and glia cells) to investigate potential pain pathways ex vivo (e.g. by using Calcium influx/microscopy).
2) (immune) cells for adoptive transfer experiments (linked to appendix 1/2)

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

To achieve our aim, we will directly kill mice/rats to obtain tissue/cells to collect materials for *ex-vivo* analysis or to collect donor cells for adoptive transfer experiments. So, no further treatments are in place.

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

We will predominantly use mice for:

1) Ex vivo analysis: Depending on the research question we can calculate the exact animals needed. For example, for neuronal cultures we can obtain approximately 120k sensory neurons per animal. If we want to measure neuronal activity ex vivo using calcium imaging, we need 15k cells per well, indicating that with one animal we can seed 8 wells. However, for microscopy analysis (e.g. if we want to investigate if mitochondria are transferred to neurons), we need 25k cells per well. So, if we want to compare control versus pro- and anti-inflammatory macrophages in triplicates we need to pool the sensory neurons of two mice.

   Based on experience from previous CCD, we expect to have ~3 experimental groups (e.g. M0, M1, M2 macrophages or neurons/glia stimulated with vehicle, stress inducers, stress-inducer + treatment) per experiment, which indicates 2 mice/exp. We expect to perform 20 experiments/year, totaling 200 mice for the 5-years period.

2) Adoptive transfer: From one animal we can obtain $10x10^6$ macrophages from the bone marrow, and $3x10^6$ T-cells from the spleen. In previous experiments we injected 30k cells/animal, and an adoptive transfer experiment normally consist of 8 mice/group (see appendix 1). This indicates that 1 mouse is enough for one behavioral experiment. However, we often want to compare adoptive transfer of male versus female mice, indicating that we need 2 mice per experiment. We expect to perform 6 adoptive transfer experiments per year, totaling 60 mice for the 5-years period

Since we will use rats for some experiments, we will calculate 10% extra rats for this appendix, indicating 20 rats for ex vivo analysis and 6 rats to collect donor cells for adoptive transfer.

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
Click or tap here to enter text.	Mouse	Utrecht University, GDL or licensed suppliers (e.g Janvier)	Adults > 7 weeks	260	Both	Both WT and GG	Various
	Rat	Utrecht University, GDL or Licensed suppliers (e.g. Charles River)	Adults >7 weeks	26	Both	WT	Various

Provide justifications for these choices

Species	We aim to use alternative methods such as in vitro cultures (see below by 3Rs), however this is not always possible since we see that some cell lines do not express all important pain receptors or have features of primary cells (e.g. responding to depolarizing agent). To understand the mechanisms of chronic pain development of resolution of pain, we need to isolate pain-relevant cells from mice/rats to study pathways ex vivo to conform the findings found in vivo in the same species. Additionally, we need mice and rats to collect donor cells for giving back to mice/rats in vivo (appendix 1/2).
Origin	Mice will be either bred at the GDL, University Utrecht or bought from licensed suppliers (e.g. Janvier). Rats will be bought as well.
Life stages	We will mainly use adult animals. If experiments show that age-specific effects exist, we may have to use one specific life stage.
Number	We expect to use 260 mice and 26 rats, see explanation by "statistical methods".
Gender	We use both males and females. If experiments show that gender-specific effects exist, we may have to use one specific gender.
Genetic alterations	We will use mice with a Cre-loxP system to delete genes in specific cells or use global knockout mice that enable us to address specific questions e.g. mitochondrial transfer. For example, recently we use LysM-Dendra mice to collect the macrophages, since those contain fluorescent mitochondria, which will help to visualize mitochondrial transfer in our ex vivo experiments. Additionally, for the T2DM model we will use mice which are transgenic for hIAPP and are obese due to an inactivating mutation in the Leptin gene (hIAPP/ObOb).
Strain	In general, mainly C56Bl/6 mice will be used. They are the most commonly used mouse strain for pain studies and most knockout mice are on this background. However, for some specific questions we may need other strains because: 1) genetic modified mice have a specific background and backcrossing would cost many mice whilst not per se needed 2) the model/purpose of a study is easier to investigate in a specific strain. For our experiments in rats, we primarily plan to use WISTAR, as the models we aim to induce are most established in this strain.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☒ Yes

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

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☒ No

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

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

Click or tap here to enter text.

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

Click or tap here to enter text.

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

Explain why these effects may emerge.

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

Click or tap here to enter text.

E. 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 F

☐ Yes > Describe the criteria that will be used to identify the humane endpoints.

Click or tap here to enter text.

Indicate the likely incidence.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

In this appendix, animals will be sacrificed without any prior intervention.

Discomfort of animals will be :
Non-recovery: 10 % (animals perfused before extraction of tissues, those animals will undergo terminal anesthesia)
Mild: 90 %

Rats will be 100% mild

G. 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	As part of our studies, we will employ a range of in vitro techniques to investigate key metabolic and neuro-immune mechanisms underlying transient and chronic pain. Currently we are expanding our research using patient-derived materials and induced pluripotent stem cells (iPSCs). For instance, with iPSC models we will generate sensory neurons, immune cells, and other relevant cell types (e.g. osteoclasts and osteoblasts) involved in pain regulation. Moreover, we will use cells stored in our freezer, if possible. For example, if we collect mouse bone marrow, the yield of macrophages is sometimes higher than needed for an experiment. Then we can store those macrophages for another upcoming experiment.
Reduction	If the use of iPSC models is successful, this could reduce the number of mice needed for ex vivo analysis. Moreover, if we culture DRG cells for ex vivo experiments we will, if practically/time-wise possible, also directly collect bone-

	marrow derived macrophages to store them for future adoptive transfer experiments or in vitro experiments. This will reduce the number of mice needed for collection of donor cells.
Refinement	Animals will be directly killed in a professional manner.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☒No

☐ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Click or tap here to enter text.

H. Re-use

Will animals be used that have already been used in other animal procedures?

☒ No > Continue with question I.

☐ 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.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Not applicable

J. Location where the animals procedures are performed

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

☒ No > Continue with question K.

☐ 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.

Click or tap here to enter text.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

Click or tap here to enter text.

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

Animals will be used to collect tissue/cells (e.g. DRG, spinal cord, immune cells) for ex vivo analysis or as donor cells for further in vivo experiments as explained in appendix 1.

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

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

Click or tap here to enter text.

☒ Yes > Will a method of killing be used for which specific requirements apply?

☐ No > Describe the method of killing.

Click or tap here to enter text.

☒ Yes > Describe the method of killing that will be used and provide justifications for this choice.

Animals will be mainly sacrificed using cervical dislocation or CO2 asphyxation. When needed, animals can be perfused with PBS or PFA after sedation or overdose of pentobarbital. For rats, we will either perform heart puncture while the animal is under anesthesia or we will sacrifice the animal through an overdose of pentobarbital.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Click or tap here to enter text.

Centrale Commissie Dierproeven

Appendix
Description animal procedures

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1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	11500
1.2	Provide the name of the licenced establishment.	UMCU, Utrecht University
1.3	List the serial number and type of animal procedure *Use the numbers provided at 3.4.3 of the project proposal.*	Serial number: 4 Type of animal procedure: Breeding of genetically altered mice with discomfort profiles

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.

To study mechanisms of (chronic) pain we make use of transgenic mice to knock-out or knock-in a gene of interest. As example we use hIAPP/Ob mice to study diabatic neuropathy. These mice become obese due to inactivating mutation in the Leptin gene. From the age of 10 months onwards these animals can have a reduced immobility and may fall over, so mice will be killed before that age to prevent severe discomfort. Currently we are breeding these mice also without the leptin gene, so that they only express the h[redacted]mportant for the formation of amyloids, which can promote nerve damage and pai[redacted]We currently are breeding these mice under "Het fokken van genetische gewijzigde muizen [redacted]2216539) but aim to do the breeding under this new CCD. Currently our other genetic modified Cre/lox mice or conditional knockout mice do not have discomfort. However, we do not know whether new CRE-LOX combinations or new genes that will be discovered in our research will give mice with discomfort. If so, we will ask for an amendment for this appendix. We will use mice on this appendix 1) with known discomfort, and/or 2) to determine genetic profile which could not be established with standard identification tissues (e.g., when a blood sample is needed for genetic profiling).

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

Breeding of mice. For each experimental set-up we will determine how many breeding rounds are needed to get the right number of experimental animals. Ear marks are obtained to test whether the pups are homozygous (HO), heterozygous (HE) or wildtype (WT) for hIAPP.

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

Statistical analysis does not play a role for the maintenance of genetic modified colonies. We will breed mice with the minimum number of mice possible. Depending on the experimental design, we will write a breeding protocol to calculate the genetically modified mice we need for experiments. The number of animals with a phenotype of mild chronic pain will (e.g. hIAPP), based on current breeding experience, not exceeds the yearly average of 100 animals requiring 5 times 100 is 500 animals for this project. In addition we expect to need 50 mice per 5 years, to get additional biomaterial in strains without a harmful phenotype (blood/ear-cut), if the genotype identification was not possible with standard procedure (e.g. because of DNA that was lost/broken down).

B. The animals

Specify the species, origin, life stages, estimated numbers, gender, genetic alterations and, if important for achieving the immediate goal, the strain.

Serial number	Species	Origin	Life stages	Number	Gender	Genetically altered	Strain
Click or tap here to enter text.	Mouse	Utrecht University, GDL or licensed suppliers (e.g Janvier)	Adults > 7 weeks	500 + 50 = 550	Both	Both WT and GG	Various

Provide justifications for these choices

Species	We will use mice (mus musculus) because these are the most commonly used species for pain research and are easier to genetically modify
Origin	Mice will be either bred at the GDL, University Utrecht or bought from licensed suppliers (e.g. Janvier).
Life stages	We will mainly use adult animals. If experiments show that age-specific effects exist, we might have to use one specific life stage.
Number	Breeding of animals is tailored to planned experiments described in appendix 1-2-3 to minimize breeding of surplus

Gender	We use both males and females. If experiments show that gender-specific effects exist we might have to use one specific gender.
Genetic alterations	We will use global knock-in (hIAPP) mice that enable us to address specific questions e.g. how hIAPP causes chronic pain & whether targeting hIAPP is a novel therapeutic approach.
Strain	C56Bl/6 background mice will be used. They are the most commonly used mouse strain for pain studies and most knockout mice are on this background.

C. Accommodation and care

Is the housing and care of the animals used in experimental procedures in accordance with Annex III of the Directive 2010/63/EU?

☒ Yes

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

Click or tap here to enter text.

D. Pain and compromised animal welfare

Will the animals experience pain during or after the procedures?

☒ No

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

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

Click or tap here to enter text.

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

Click or tap here to enter text.

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

Click or tap here to enter text.

Explain why these effects may emerge.

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

Click or tap here to enter text.

E. 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 F

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

As described above, we know that hIAPP/Ob mice experience hypersensitivity (hIAPP/Ob). We will monitor their wellbeing and stop the breeding in there is a clear increase in discomfort phenotype, like;
- Animals that show spontaneous pain behaviour in the cage (looking at grimace, fur, posture)
- Animals that cannot walk normally (visible limping)
- Animals with altered social and grooming behaviour
- Abnormal vocalization when animals are touched or handled

Indicate the likely incidence.

We don't expect to reach the HEP, since we have experience in breeding this line and kill the mice before the 10 months to prevent severe suffering.

F. Classification of severity of procedures

Provide information on the experimental factors contributing to the discomfort of the animals and indicate to which category these factors are assigned ('non-recovery', 'mild', 'moderate', 'severe'). In addition, provide for each species and treatment group information on the expected levels of cumulative discomfort (in percentages).

If discomfort of animals bred exceeds the category "mild", breeding will be discontinued. If additional material for genotyping is necessary (e.g. taking blood or an extra ear-cut), it will also be classified as "mild".

G. 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	Use of in vitro models (cell lines, iPSCs) if possible to replicate aspects of the phenotype.
Reduction	Use a small breeding to maintain the line and avoid overbreeding.
Refinement	Breed heterozygous animals with milder phenotypes rather than homozygotes if animals are found to have a harmful phenotype.

Are adverse environmental effects expected? Explain what measures will be taken to minimise these effects.

☒ No

☐ Yes > Describe the environmental effects and explain what measures will be taken to minimise these effects.

Click or tap here to enter text.

H. Re-use

Will animals be used that have already been used in other animal procedures ?

☒ No > Continue with question I.

☐ 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.

Click or tap here to enter text.

I. Repetition

Explain for legally required animal procedures what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, describe why duplication is required.

Not applicable

J. Location where the animals procedures are performed

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

☒ No > Continue with question K.

☐ 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.

Click or tap here to enter text.

3. End of experiment

K. Destination of the animals

Will the animals be killed during or after the procedures?

☐ No > Provide information on the destination of the animals.

Click or tap here to enter text.

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

After the breeding, mice will be used for appendix 1 or 2. Animals will be used to collect tissue/cells (e.g. DRG, spinal cord, immune cells) for ex vivo analysis or as donor cells for further in vivo experiments as explained in appendix 1 and 2. Animals (with no discomfort) that cannot be used will be listed on ATEX.

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

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

Click or tap here to enter text.

☒ Yes > Will a method of killing be used for which specific requirements apply?

☐ No > Describe the method of killing.

Click or tap here to enter text.

☒ Yes > Describe the method of killing that will be used and provide justifications for this choice.

Animals will be mainly sacrificed using cervical dislocation or CO_2 asphyxation.

If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

Click or tap here to enter text.

ADVIES PROJECTVOORSTEL DIERPROEVEN

A. Algemene gegevens over de procedure

1. Aanvraagnummer : AVD11500202519049
2. Titel van het project : Deciphering neuro-immune interactions in pain regulation
3. Titel van de NTS : Bestuderen welke rol onze eigen afweer heeft in het ontwikkelen van (chronische) pijn

4. Type aanvraag:
   - ☒ nieuwe aanvraag projectvergunning
   - ☐ wijziging van vergunning met nummer :

5. Contactgegevens DEC

Naam DEC	: DEC Utrecht
Telefoonnummer contactpersoon	: 06-31118069
Emailadres contactpersoon	: dec-utrecht@umcutrecht.nl

6. Adviestraject (data dd-mm-jjjj):
   - ☒ ontvangen door DEC: 11-07-2025
   - ☐ aanvraag compleet:
   - ☒ in vergadering besproken: 16-07-2025 (onbeoordeelbaar) en 03-09-2025 en 01-10-2025
   - ☐ anderszins behandeld:
   - ☒ termijnonderbreking(en) van / tot: 22-07-2025 / 21-08-2025 en 08-09-2025 / 19-09-2025
   - ☒ besluit van CCD tot verlenging van de totale adviestermijn met 15 werkdagen: 22-08-2025
   - ☐ aanpassing aanvraag:
   - ☒ advies aan CCD: 09-10-2025

7. De aanvraag is afgestemd met de IvD en deze is hiermee akkoord.

8. Eventueel horen van aanvrager
   - Datum: 03-09-2025
   - Plaats: Online via Teams
   - Aantal aanwezige DEC-leden: 5
   - Aanwezige (namens) aanvrager: Onderzoeker
   - Gestelde vragen en verstrekte antwoorden: De DEC heeft de onderzoekers o.a. gehoord over de omvang van het project, de go/go no momenten en de criteria, de ongeriefsinschatting, de volgorde van de modellen en het aantal dieren bij de fok. Hieruit zijn onderstaande vragen, zoals vermeld bij punt A9, voortgekomen, die schriftelijk aan de onderzoekers werden voorgelegd.
   - Het horen van de aanvrager heeft geleid tot aanpassing van de aanvraag.

9. Correspondentie met de aanvrager

- Datum vragen: 08-09-2025
- Datum antwoord: 19-09-2025
- Strekking gestelde vragen en antwoorden:
  Kunt u specifieker zijn, en daarmee beter navolgbaar maken, wat er met welk dier gaat gebeuren, hoe vaak en hoe lang deze blootgesteld gaat worden aan wat, wanneer wel en wanneer niet en op basis waarvan?
  We agree with the need for details, and clearer outline of the procedures to ensure it is clear what an animal can undergo and the impact it would have on their wellbeing. To address this we have changed figure 3 and made several possible options that exist withing each DAP (see figure 2 appendix 1 and 2). For convenience we have incorporated these figures outlining the procedures below.
  Using Appendices 1–3, we distinguish between three types of studies: transient pain models (Appendix 2), chronic pain models (Appendix 1), and studies involving tissue collection without behavioral assessments (Appendix 3). In Appendices 1 and 2, the new figure 2 illustrates the general experimental setup with indication what procedure an animal can undergo (see also figure below). Importunately, mice undergo either 1 transient pain model, designed to assess whether an immune cell or protein contributes to pain resolution, or 1 chronic pain model to evaluate the effects of targeting specific immune cells or proteins on pain maintenance. In all cases we follow a unified strategy involving perturbing the immune system or proteins via different methods (genetic or pharmacological) and monitoring resultant pain behaviors and/or tissue responses.
  For transient models (DAP2), the goal is to determine whether a particular immune cell or protein prevents resolution, while in chronic models, we aim to test how interventions can promote resolution and pain recovery. To improve clarity, we have added a new Figure 2 in Appendix 1 and 2 for the procedures mice can undergo, and have revised Figure 3 in the main proposal for better visualization of the project strategy for clear go/no-go criteria.
  In practice, we will analyze one pain model at a time, either a transient or a chronic one within Objective 1, and only 1 chronic for objective 2. The number of behavioral measurements is restricted for ethical and feasibility reasons. For instance, in a transient model, we measure pain-associated behaviors daily at the expected peak and during the resolution period, typically over a maximum of 7 days, and in some models max 14 days. For models, where full resolution take longer, measurements in the first week will be less to be able to measure daily during resolution in the second week. In chronic models, measurements may be less frequent (e.g once a week) but will extend over longer periods, especially around intervention points where we will perform daily measurements. In some other cases our outcome is something else than pain behaviours, e.g. changes in immune cells in the DRG. In those cases we limited the number of pain measures, to e.g. baseline, moment to measure correct development of model, and at the end prior to tissue harvest.
  New figure details procedure animal can undergo. DAP1. DAP2.
  Dit is nu voor de DEC bijvoorbeeld onduidelijk op plaatsen waar u de term '*Sometimes*', '*Often*', '*In (some) case*', '*if*' gebruikt.

We reviewed the entire proposal and removed ambiguous conditional terms ("sometimes," "often," "in (some) cases," "if") wherever a precise, fixed procedure could be stated. These edits are highlighted in the revised document.
Where conditional language remains, this reflects the nature of hypothesis-driven, exploratory fundamental research: outcomes at early stages determine the subsequent, predefined experimental path (go/no-go decision points). To minimize ambiguity we have made these decision rules explicit where possible (see revised Figure 3 and the detailed procedure figures). For each conditional step we now state the specific experimental trigger and the exact consequence (e.g., "if X produces ≥30% change in behavior and consistent target engagement, then proceed to Y; if not, stop this line").

Wat is de volgorde van de modellen? Een beslisboom betreffende de keuze van de verschillende pijnmodellen zou wenselijk zijn. Onder welke voorwaarden, criteria, go/ no-go momenten wijkt u uit naar een ander model?

Thank you — we agree this needed clearer ordering and decision rules. We revised the text below Tables 1 in DAP1 & DAP2 why we use specific models (highlighted in yellow) and substantially updated Figure 3 (see below for new figure) to provide an explicit decision tree showing selection criteria, and go/no-go thresholds.
Key changes made:
-Specified preferred starting models for each question (based on prior data and literature) in the text beneath Tables 1 in DAP1 & DAP2.
-Defined objective, measurable go/no-go criteria (e.g., ≥30% change in pain-related behavior combined with target engagement in ex vivo readouts) that determine whether a candidate advances.
-Clarified the branching logic: positive results in a model motivate testing the same intervention in a related model of different etiology (e.g., OA → chemotherapy-induced neuropathy) to assess generalizability; negative results terminate that line or redirect it to an alternative hypothesis/model.
-Added examples in the text to illustrate how decisions will be made (e.g., if complement inhibition reduces OA pain and transcriptomic/clinical data implicate complement in neuropathy, we will next test complement inhibition in a chemotherapy-induced neuropathy model). Updated figure 3.

Wilt u verduidelijken, wanneer u kiest voor een model in dezelfde categorie en wanneer in een andere categorie?

We will primarily test neuro-immune interactions within the same pain category (inflammatory, bone, or neuropathic) that matches the human data or biological rationale for the candidate. We will move to a model in a different category only to assess generalizability when predefined success criteria are met in the original category. Concretely:
-Primary criterion for staying within the same category: the candidate shows mechanistic evidence in the initial model (preclinical go criteria: ≥30% improvement in pain behavior plus consistent ex vivo target engagement).
-Criterion for testing a different category: the candidate meets the primary go criteria and either (a) human or other available data indicate the pathway is also implicated in the other

category, or (b) demonstration of efficacy in a second, mechanistically related model is required to establish broader relevance.
-If the candidate fails the primary go criteria, we will not test it in other categories (no-go). When moving categories, model choice will be hypothesis-driven and justified (e.g., OA → chemotherapy-induced neuropathy if transcriptomics or clinical data implicate complement in both).
Kunt u entree-criteria geven van stoffen die u door de farmaceut worden aangeleverd voor uw dierexperimenten?
We appreciate the opportunity to clarify. We will not act as a contract research organization (CRO) testing industry compounds. We only use a drug for objective 2 of a pharmaceutical company because our research points towards that factor X should be targeted, See figure 3. So only in case we identify a target where a company has an drug/antibody that effectively blocks the target X, we may use this. So the entry-criteria are based on our go/no go of our objectives and not because a pharmaceutical company wants to test something.
Wilt u een afbakening geven wanneer de cyclus stopt of wanneer u genoeg modellen hebt getest? Op basis waarvan besluit u dat? Om hoeveel targets gaat het?
*In general most work is aimed to identify **mechanistically** how a cell/protein is involved in pain resolution or chronification and we will use just one model. For example if we want to fully understand how LAIR1 effects pain resolution, the majority of the work will be done in transient pain models, until we have resolved the mechanism. We will then only test once in a chronic pain model whether it may have therapeutic benefit. Based on this we expect not to test more than 1-2 targets a year for proof-of-concept for a therapy. We adjusted figure 3 where this should become more clear.*
De DEC heeft de ongeriefinschatting nog niet beoordeeld, omdat er te veel onduidelijk is over wat er met welk dier gaat gebeuren.
Thank you. We appreciate the committee's concern. We have therefore now made clear figures of the procedures that an animal can go through. We will only use mild/moderate models as explained in the DEC meeting (Sept. 3rd, 2025). In support that these models are moderate, we checked with the IVD and the last 5-10 years we never had to report severe suffering in the described models. In all models we monitor and have defined humane endpoint to prevent severe discomfort. Also the number and frequency of addition procedure to the pain model are restricted to stay within moderate discomfort. By providing examples of experimental set-ups (adjusted text, new figure 2 in appendix 1 and appendix 2) we hope it is more clear for the committee how a general experiment is performed.

Projectvoorstel
3.1 Achtergrond
Kunt u beschrijven welke factoren een rol spelen bij de communicatie tussen immuuncellen en neuronale cellen/structuren?
In the background we explain which factors are important in this communication based on results that we achieved the last 5 years. We identified inhibitory receptor as important factor, as well as complement, IgG, and IAPP. We also highlight those in figure 1. But the main point of

our research is to identify the way of neuro-immune communication. We adjusted the background.

U schrijft nu over een verband tussen pijn en het immuunsysteem. Kunt u een grijpbare hypothese geven?

We have completely rewritten the goal section to make our hypothesis and the objectives to test this hypothesis more clear and tangible.

3.2 Doel

U wilt 3 soorten pijn bestuderen, maar die komen niet terug bij/ komen niet overeen met de *objectives*. Wilt u dat aanpassen om het verband tussen beide helder en navolgbaar te maken?

Apologies for not being clear here. We added paragraphs in e.g. 3.1 (background) and 3.2.2 (project feasibility) why we study three different types of chronic pain.

For example we now state:

The justification to focus on inflammatory-, neuropathic- and bone-pain is that these conditions are clinically relevant. In addition heterogeneity of clinical pain: Inflammatory, neuropathic and bone pain have distinct initiating causes and clinical courses. Using multiple models ensures our findings are relevant across these clinically diverse conditions rather than restricted to a single etiology. Although different insults can converge on common pathways, they also engage distinct neuro-immune triggers (e.g., complement and autoantibodies in OA, metabolic aggregates in diabetic neuropathy). Comparing models lets us separate convergent, generalizable mechanisms of resolution from etiology specific pathways. Demonstrating that a neuro-immune interaction (e.g., macrophage mitochondrial transfer, inhibitory receptor signaling, complement activation) is necessary and/or sufficient across multiple models strengthens causal inference and supports that the mechanism is broadly relevant to chronic pain.

3.2.2 Feasibility:

Wilt u de praktische haalbaarheid beschrijven van concrete doelen en eindpunten?

We have adjusted figure 3 to add more clear "go/no-go" moments.

Hoeveel aantoonbare ervaring hebt u met ieder van de grijze modellen? Kunt u dit toelichten? Kunt u duiden hoe het door u geobserveerde ongerief zich verhoudt tot hetgeen beschreven is in de literatuur voor de grijze modellen? Hoe verklaart u dat?

We have ample experience with all the models indicated in grey models, which can be verified in our publications. We make this now clear in the DAPs including the discomfort. However, we understand we gave the impression that we could use any model. Therefore we have gone through all models and removed various grey models. In case our work needs those models we will apply for an amendment.

3.3 Belang: Kunt u beschrijven welk model u verbindt aan welke theorie? Kunt u uw theoretische werkhypothese beschrijven? Op welk model of op welk celtype wilt u wat testen en waarom?

In appendices 1 and 2 we added more information on the models, and rationale / input we use to choose for a certain model.

3.4 Strategie

Onder figuur 1 staat de zin: *As one type of pain cannot be represented by a single model, we need to have the ability to choose a model depending on the current literature, discussions with our scientific collaborators and industry-based partners, to ensure…* Wat is er beschreven en welke richtingen geven de literatuur, discussies met 'scientific collaborators' en 'industry-based partners' aan het model, dat u gaat kiezen? Wat gaat u doen, hoe en wanneer en waarom?

Indeed this sentence we wrote under figure 1 of Appendix 1 and 2 and removed the industry partners, given that it gave the wrong impression. Model choice is evidence driven and primarily guided by scientific data (literature, our prior experiments, and human patient findings) and by consultation with our academic collaborators. Industry input is not used to select animal models; industry is consulted only if an externally developed compound exists for a validated target

Uw opmerking '*Dieronderzoek is noodzakelijk*' wordt niet ondersteund door de DEC, maar de DEC ziet liever een uitleg waarom het niet anders kan.

Apologies for not being clear. We reworded the text to explain why we cannot do without animals to investigate mechanisms that are important for neuro-immune communication. We explained this in 3.3.1, and reads:

We will use in vitro models (e.g neuro-immune cultures), human patient materials (e.g. blood, cerebrospinal fluid, human patient skin for e.g. immunostaining including nerve innervation), and induced pluripotent stem cells (iPSCs), but these systems do not fully capture the complexity of the pain system. Therefore, animals play an indispensable role in achieving our scientific goals. We focus inflammatory-, neuropathic- and bone-pain since these conditions are clinically relevant, which are per condition more broadly explained in appendix 1.

Pain is a complex, system-wide process involving intricately connected neuronal and immune networks that cannot be fully recapitulated in vitro. Animal models allow us to:

-Investigate pain resolution and pain chronification in the context of an intact nervous and immune system, capturing dynamic, cell-to-cell and tissue-to-tissue interactions.

-Experimentally manipulate genetics, immune cell subsets, and metabolic pathways in vivo to determine causal relationships.

-Translate findings into proof-of-concept therapeutic strategies prior to clinical application, ensuring both efficacy and safety.

U geeft nu go/ no-go momenten aan het einde van uw project in flow chart figuur 3 in rood. Waar leiden die tot/ naar toe? Kunt u meer tussentijdse go/ no-go momenten geven om keuzes te maken en de bijbehorende criteria geven?

We adapted the figure ad text to explain the end of the project better. Please see new figure in response above.

U hebt geschreven dat de go is >15% effect of >30% toename en de no-go is geen effect of toename. Kunt u toelichten wat er gebeurt tussen een klein beetje en 15% of 30%? Kunt u een verklaring geven over het gekozen verschil tussen >15% *in vitro* en >30% *in vivo*?

For in vivo experiments we use 30% cut-off because we want to have clear effect sizes and given that a similar percentage is used in the clinic, where pain VAS score have to reduce with 30% or 2 VAS points to consider it clinically relevant (PMID: 32520773/ 11690725). For our

mice experiments this indicates that e.g. heat sensitivity reduction in a model is 4 seconds (8 seconds at baseline to 4 seconds during pain), treatment has to at least reduce it with 1.2 seconds (30% of 4 seconds difference). For the in vitro assays, we take a smaller percentage because in these studies we only asses one aspect, e.g. neuronal functioning, effect immune cells, whilst in vivo it is the combination of all. Therefore we think, and what we have observed in our previous work, that e.g. co-culturing neuronal cultures with anti-inflammatory macrophages, reduction in neuronal activity is more subtle. So based on these observations we determined >15% as a "GO" for in vitro experiments.

Bijlage 1

A. Experimentele aanpak en primaire uitkomstparameters

Wilt u beargumenteren waarom u voor het voetzool model kiest? Is dit het enige geschikte model? Waarom is er geen ander subcutaan infectiemodel geschikt?

We are studying inflammation (not infection) and inject inflammatory agents into the hind paw (intraplantar) to mimic a local inflammatory response and where pain-behavioral readouts are well employable. Subcutaneous injection at other sides are not readily accessible for e.g. testing mechanical hypersensitivity. Additionally alternatives locations (e.g. the back) is not accessible to measure pain, due to hair. For these reasons intraplantar models are widely used in the pain field to study chronic pain.

Kunt u criteria geven op basis waarvan u afwijkt van het zwarte model en kiest voor het grijze model?

We removed various grey models and now indicate why/how we choose a model

Kunt u in tabel 1 een kolom toevoegen met de uitleg, waarvoor elk model geschikt is en wanneer u naar dat model uitwijkt?

We added this info below the table.

F. Classificatie van ongerief: Hoeveel aantoonbare ervaring hebt u met ieder van de grijze modellen? Kunt u dit toelichten? Kunt u duiden hoe het door u geobserveerde ongerief zich verhoudt tot hetgeen beschreven is in de literatuur voor de grijze modellen? Hoe verklaart u dat?

See response above.

Bijlage 4

B. De dieren: Bij 'Number' schrijft u 'Depending on the research question'. Wilt u uitzoeken met de IvD (en de CCD) of u hier de surplus dieren van de fok met ongerief of ook de dieren voor uw experimenten moet vermelden?

We added the possibility that we generate new CRE-LOX lines with potential discomfort upcoming years. However we now removed this text from appendix 4 and we will write an amendment if we generate a new line with possible discomfort. The numbers that are now calculated are only for the hIAPP mice. We adjusted the numbers also in the NTS.

- De antwoorden hebben geleid tot aanpassing van de aanvraag.

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

B. Beoordeling (adviesvraag en behandeling)

1. Het project is vergunningplichtig (dierproeven in de zin der wet).
2. De aanvraag betreft een nieuwe aanvraag.
3. De DEC is competent om hierover te adviseren.
4. Er zijn geen DEC-leden betrokken bij het betreffende project.

C. Beoordeling (inhoud):

1. De aanvraag is toetsbaar en heeft voldoende samenhang. Dit is een vervolgproject op aanvraag *Unravelling neuro-immune interactions governing mitochondrial health in pain* (AVD11500202010805). Het onderzoek betreft de rol van het immuunsysteem en interacties tussen immuuncellen en neuronen in het ontstaan, het bestendigen en het weer oplossen van pijn. Men wil in muizen en ratten mechanismen bestuderen van het ontstaan en het weer voorbijgaan van transiënte en chronische pijn en ook de processen bestuderen die een rol spelen bij de overgang van transiënte naar chronische pijn. Hiertoe worden in verschillende diermodellen, naar analogie met belangrijke oorzaken van humane pijn, botpijn, neuropatische pijn en ontstekingspijn geïnduceerd. Mede op basis van eerdere resultaten wordt verwacht dat het ontstaan en voorbijgaan van deze drie verschillende vormen van pijn overeenkomsten en verschillen zullen vertonen. Stoffen of behandelingen die in een relevant model effectief bevonden worden, zullen vervolgens in een ander model voor dezelfde vorm van pijn en eventueel in nog een model voor een andere vorm van pijn worden getest om vast te stellen of ze alleen actief zijn in één specifiek diermodel, of actief zijn tegen een specifieke vorm van pijn, of meer algemeen actief zijn tegen verschillende vormen van pijn.

   De aanvraag is volgens de DEC ruim geformuleerd maar navolgbaar en de samenhang tussen de subdoelen, het begrijpen van het ontstaan en het vinden van aangrijpingspunten voor het ontwikkelen van rationele therapieën voor bestrijding van chronische pijn, is voldoende helder waardoor deze aanvraag een project betreft (3A), al zijn de activiteiten op afdelingsniveau beschreven.

2. Voor zover de DEC bekend, is er geen mogelijk tegenstrijdige wetgeving die het uitvoeren van de dierexperimenten in de weg zou kunnen staan.

3. De in de aanvraag aangekruiste doelcategorieën sluiten aan bij de hoofddoelstelling(en) al gaat het in dit stadium van onderzoek met name om fundamenteel onderzoek dat uiteindelijk transleerbaar kan zijn.

   Belangen en waarden

4. Het directe doel van het project is te onderzoeken hoe de interactie tussen zenuwen en afweersysteem pijn beïnvloedt en het falen hiervan chronische pijn veroorzaakt, waarbij men wil aantonen dat beïnvloeding van deze interactie pijn kan verlichten. Het uiteindelijke doel van het

project is meer inzicht verkrijgen in processen die een rol spelen bij het ontstaan en weer verdwijnen van pijn en van processen die een rol spelen bij de overgang van transiënte naar chronische pijn. Daarbij wordt gezocht naar aanknopingspunten voor nieuwe vormen van pijnbestrijding en zal de effectiviteit van modelstoffen voor het bestrijden van verschillende vormen van pijn worden bestudeerd. De DEC is van mening dat er een duidelijke relatie is tussen het directe en het uiteindelijke doel, en dat het doel gerechtvaardigd is in de context van het neuro-immunologisch onderzoeksveld en de behoeften vanuit de gezondheidszorg en patiënten met (chronische) pijn.

5. De belangrijkste belanghebbenden in dit onderzoeksproject zijn: proefdieren, onderzoekers, samenwerkingspartners, gezondheidszorg en patiënten met chronische pijn. De muizen en ratten hebben er als proefdieren een groot belang bij gevrijwaard te blijven van de experimenten, het ongerief en de vroegtijdige dood. De onderzoekers, en de wetenschappelijke (internationale) samenwerkingspartners, hebben juist belang bij positieve resultaten uit het onderzoek naar aanknopingspunten voor nieuwe mogelijkheden voor effectieve pijnbestrijding. Door het delen van de vooralsnog fundamentele onderzoeksresultaten kunnen andere (internationale) onderzoekers ook verder met de bevindingen, met name binnen het Europese pijnconsortium waarmee nauw samengewerkt wordt. Samenwerkingspartners zoals farmaceutische of biotechnologische bedrijven kunnen commerciële belangen hebben maar in de uiteindelijke afweging kent de DEC daar geen gewicht aan toe. De gezondheidszorg en veel (toekomstige) patiënten met chronische pijn, die vaak slecht of niet behandelbaar is, hebben eveneens een groot belang bij de onderzoeksresultaten doordat mogelijk bijgedragen kan worden aan een effectievere pijnbestrijding en de kwaliteit van leven van patiënten verhoogd kan worden.

6. De aanvrager geeft aan dat nadelige effecten op het milieu kunnen ontstaan doordat enkele studies naar chemisch geïnduceerde pijn (zoals CIBP) een behandeling met chemotherapeutica vereisen. De behandelde dieren worden echter in aparte sociale kooien geplaatst en afval volgens de richtlijnen afgevoerd. De DEC ziet daarom geen aanleiding om aan te nemen dat zich na de te nemen voorzorgsmaatregelen nadelige effecten op het milieu zullen voordoen.

Proefopzet en haalbaarheid

7. De kennis en kunde van de zeer ervaren onderzoeksgroep met de verschillende pijn- en diermodellen en andere betrokkenen bij de dierproeven zijn voldoende gewaarborgd en dragen eraan bij dat de doelstellingen behaald kunnen worden, dat aan de 3V-beginselen voldaan kan worden en dat voorkomen kan worden dat mens, dier en milieu negatieve effecten ondervinden als gevolg van de dierproeven.

8. Het project is ruim omschreven maar goed opgezet, en met de drie bijlagen en een aparte fokbijlage ook duidelijk gestructureerd. De voorgestelde experimentele opzet en uitkomstparameters sluiten logisch en helder aan bij de aangegeven doelstellingen. De go/no

go's zijn helder beschreven en de onderzoekscyclus is voldoende begrensd. Verschillende modellen worden als 'startmodel' toegepast voor het bestuderen van verschillende specifieke vormen van pijn. Er zijn duidelijke criteria om eenzelfde stof of behandeling te testen in één aanvullend model in dezelfde categorie (ter validatie van de specificiteit voor een bepaalde vorm van pijn) en aanvullende criteria om eenzelfde stof of behandeling te testen in één aanvullend model in een andere pijncategorie (om vast te stellen of het vastgestelde specifieke effect ook generaliseerbaar is naar andere vormen van pijn). Het is navolgbaar en controleerbaar wat met elk individueel dier gebeurt en in welk model, waardoor ook de werkprotocollen helder kunnen zijn. De gekozen strategie en experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het project.

Welzijn dieren

9. Er is sprake van de volgende bijzonderheden op het gebied van categorieën van dieren, omstandigheden of behandeling van de dieren:
   ☐ Bedreigde diersoort(en) (10e lid 4)
   ☐ Niet-menselijke primaten (10e)
   ☐ Dieren in/uit het wild (10f)
   ☐ Niet gefokt voor dierproeven (11, bijlage I EU richtlijn)
   ☐ Zwerfdieren (10h)
   ☐ Hergebruik (1e lid 2)
   ☐ Locatie: buiten instelling vergunninghouder (10g)
   ☒ Geen toepassing verdoving/pijnbestrijding (13)
   ☐ Dodingsmethode niet volgens bijlage IV EU richtlijn (13c lid 3)

   De dieren krijgen geen pijnmedicatie toegediend, tenzij pijnstilling geen invloed heeft op het experiment zoals anesthesie voor verschillende invasieve ingrepen en perioperatieve pijnbestrijding voor het CIBP model. De DEC beseft dat onthouding van pijnmedicatie extra ongerief veroorzaakt voor de dieren maar ziet de noodzaak hiervan in omdat pijn (en de integrale reacties van het organisme op pijn) de primaire experimentele parameter vormt in dit onderzoek. De keuze hiervoor is voldoende onderbouwd en de aanvrager voldoet aan de in de Wet op de dierproeven, voor de desbetreffende categorie, genoemde beperkende voorwaarden.

10. De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de EU richtlijn. Meerdere dieren worden in een kooi geplaatst of waar mogelijk wordt gebruik gemaakt van een buddy waardoor individuele huisvesting wordt voorkomen. Men maakt zoveel mogelijk gebruik van automatisch monitoren in een thuiskooi.

11. Het cumulatieve ongerief als gevolg van de dierproeven is met 'matig' realistisch ingeschat en geclassificeerd. De meeste dieren worden gebruikt in diermodellen waarbij chronische pijn (neuropathie, botpijn of ontstekingspijn) opgewekt wordt. Per dier wordt maximaal één pijnmodel gebruikt. De DEC heeft uitgebreid gediscussieerd over de vraag of het cumulatief ongerief voor een deel van de dieren uiteindelijk toch als ernstig zou moeten worden

geclassificeerd. De onderzoekersgroep heeft ruime ervaring met de diermodellen, waaronder modellen waarbij ontsteking in de voetzool/poot wordt opgewekt. In hun eerdere vergelijkbare projecten is nooit ernstig ongerief opgetreden, mede door monitoring en toepassing van de humane eindpunten zoals ten aanzien van gewichtsverlies waardoor ernstig ongerief wordt voorkomen. De betrokken IvD bevestigt dat er de afgelopen vijf jaar geen ernstig ongerief is opgetreden, mede na overleg met de onafhankelijke dierverzorgers. Middels audits van de IvD zal het welzijn van de dieren nader worden geborgd. Als ernstig ongerief onverhoopt toch geconstateerd wordt, zal het betreffende diermodel direct gestopt worden en zal daarvoor een nieuwe vergunning bij de CCD worden aangevraagd.

In de discussie over de mate van ongerief heeft de DEC heeft zich afgevraagd of en waarom er geen ander subcutaan infectiemodel voor het voetzoolinfectie-experiment mogelijk is. De redenen om dit goed gevalideerde model in te zetten zijn door de onderzoekers in voldoende mate onderbouwd en daarbij is volgens de DEC voldoende toegelicht dat de in dit onderzoek voorgestelde (en eerder door de aanvragers al toegepaste) injecties van Complete Freund's Adjuvant in de voetzool geen ernstig ongerief veroorzaken,

Ongerief wordt ook veroorzaakt door herhaalde gedragstesten. De dieren ondergaan maximaal vier gedragstesten per dier waarbij de aard van de gedragstesten afhankelijk is van de onderzoeksvragen. De DEC vindt het zorgvuldig dat het aantal gedragstesten afgebakend is dat de keuze voor de vier testen in de werkprotocollen vermeld wordt.

Tot slot worden voor dit project genetisch gemodificeerde dieren gefokt (bijlage 4). De onderzoekers zullen een wijziging indienen als een nieuwe muizenlijn met mogelijk ongerief gefokt gaat worden.

12. De integriteit van de dieren wordt met name fysiek aangetast door het experimenteel opwekken van verschillende vormen van pijn met chronische pijn als gevolg en de daarop volgende (gedrags)testen.

13. De humane eindpunten, met een onderscheid tussen algemene en modelspecifieke eindpunten zijn voor iedere bijlage dierproeven (bijlage 1 en 2) goed gedefinieerd en het percentage dieren dat naar verwachting een humaan eindpunt bereikt, zoals gewichtsverlies, infectie of verlamming, is goed ingeschat. Voor het verkrijgen van de donorcellen en cellen voor *ex vivo* analyse (bijlage 3) worden geen humane eindpunten verwacht. Verder worden muizen voordat zij de leeftijd van tien maanden bereiken gedood om te voorkomen dat een mogelijk HEP in de fok (bijlage 4) wordt bereikt.

3V's

14. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn voor onderzoek naar de complexe neuro-immuuninteractie en het ontstaan en genezen van (chronische) pijn.

15. Het aantal te gebruiken dieren is realistisch ingeschat en er is een heldere strategie, zoals vooraf i*n vitro* studies, het minimaliseren van controlegroepen en de pijndatabase, om ervoor te zorgen dat tijdens het project met het kleinst mogelijke aantal dieren wordt gewerkt waarmee nog een betrouwbaar resultaat kan worden verkregen. Tevens is het fokbeleid gericht op het minimaliseren van fokoverschot.

16. Het project is in overeenstemming met de vereiste van verfijning van dierproeven en het project is zodanig opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Zo is de duur van de experimenten zo kort mogelijk en maakt men gebruik van thuiskooimonitoring.

17. Er is geen sprake van wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Dieren van beide geslachten zullen worden ingezet. Als later in het project blijkt dat bepaalde mechanismen in een van beide seksen niet werken, dan wordt in goed overleg met de IvD verdergegaan met één sekse om onnodig gebruik van dieren te voorkomen.

19. Alle dieren worden in het kader van het project gedood. Van de meeste dieren worden weefselmonsters of cellen voor *ex vivo*-analyse of als donorcellen voor verdere *in vivo*-experimenten verzameld. Het weefsel van de overige dieren wordt op het ATEX-platform geplaatst. De dieren worden op een passende wijze, in overeenstemming met bijlage IV van de EU richtlijn, gedood.

20. De vraag over hergebruik is niet van toepassing omdat de dieren gedood worden in het kader van het experiment.

NTS

21. De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd.

D. Ethische afweging

1. De morele vraag die de DEC dient te beantwoorden is of het belang van dit onderzoek, namelijk het ontrafelen welke processen en factoren een rol spelen bij inductie en het herstel van verschillende vormen van pijn en bij de overgang van transiënte naar chronische pijn, de

onvermijdelijke aantasting van het welzijn en de integriteit van de gebruikte proefdieren rechtvaardigt.

2. Er vindt een aantasting van welzijn en integriteit van de proefdieren plaats (5230 muizen en 606 ratten), met terminaal, licht en met name matig ongerief.
   Indien de hierboven genoemde doelstellingen behaald worden, dan zal dit project er toe bijdragen dat uitgebreid inzicht verkregen wordt in processen die een rol spelen in verschillende vormen van pijn en in aangrijpingspunten voor pijnbestrijding van die specifieke vormen van pijn en van (chronische) pijn meer in het algemeen. Deze kennis is noodzakelijk om (chronische) pijn in de toekomst op een rationele wijze effectief te kunnen behandelen. Dit is relevant voor de grote populatie pijnpatiënten aangezien voor een deel van hen nog geen aangrijpingspunten voor hun chronische pijn bekend zijn en voor een ander deel kunnen de onderzoeksresultaten potentiële verbeteringen in hun pijnbehandeling opleveren. Het is aannemelijk dat de fundamentele doelstelling behaald wordt, evenals de uiteindelijke transleerbaarheid van de resultaten, mede gezien de inbedding van het onderzoek en de aard van de samenwerkingspartners. Daarvoor is de inzet van proefdieren noodzakelijk, maar de onderzoekers doen al het mogelijke om het ongerief voor de dieren en het aantal dieren tot een minimum te beperken en hebben zorgvuldige maatregelen genomen om ernstig ongerief te vermijden.

3. Op grond van het bovenstaande is de DEC van oordeel dat onderzoek naar hoe de neuro-immuuninteractie pijn beïnvloedt en het falen hiervan chronische pijn veroorzaakt, een belang vertegenwoordigt en dat dit belang opweegt tegen de aantasting van het welzijn en de integriteit van de proefdieren. De relatie tussen het directe en het uiteindelijk doel is voldoende helder. Het is aannemelijk dat de directe doelstelling behaald zal worden. De commissie is overtuigd van de kwaliteit van het werk van de aanvrager. De aanvrager heeft voldoende aannemelijk gemaakt dat er geen geschikte vervangingsalternatieven zijn, dat het doel niet met minder dieren behaald kan worden, dat de gebruikte aanpak de meest verfijnde is en dat er geen sprake zal zijn van onbedoelde negatieve effecten voor mens, dier en milieu als gevolg van de dierproeven. Het gebruik van de proefdieren zoals beschreven in de aanvraag is daarmee gerechtvaardigd.

E. Advies

1. Advies aan de CCD

☒ De DEC adviseert de vergunning te verlenen.

☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden.

- ☐ Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
- ☐ Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
- ☐ Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten...

☐ De DEC adviseert de vergunning niet te verlenen vanwege:

☐ De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:…
☐ De volgende doorslaggevende ethische bezwaren:…
☐ De volgende tekortkomingen in de aanvraag:…

2. Het uitgebrachte advies is gebaseerd op consensus.

3. Er zijn geen knelpunten/dilemma's naar voren gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies.

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD11500202519049

Datum 17 oktober 2025
Betreft Aanhouden aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 11 juli 2025 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Deciphering neuro-immune interactions and metabolism in pain regulation". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD11500202519049.

In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Niet technische samenvatting
- In de tekst "fate animals kept alive" geeft u aan dat dieren waarvan geen weefsels worden verzameld zoveel mogelijk worden hergebruikt. In de tabel "Fate of animals kept alive" blijkt echter niet dat dieren worden hergebruikt.
Kunt verduidelijken of dieren worden hergebruikt of niet?
- Kunt u de verwijzing naar het centrum voor proefdiervrije biomedische translatie verwijderen, zodat de anonimiteit gewaarborgd kan worden?
- Kunt u de NTS indienen in het verplichte Excel format?

Onduidelijkheden
- In bijlage 1, onder F (ongerief) beschrijft u dat 100 muizen en 100 ratten gebruikt worden voor training en derhalve terminaal ongerief ondergaan. In de tekst hieronder beschrijft u echter dat voor de ratten 20% licht ongerief zal ervaren en 80% matig ongerief. Kunt u deze inconsistentie oplossen?
- Tabel 5 in bijlage 1 is onduidelijk. Kunt u aangeven wat de grijze en zwarte vlakken betekenen?
- In bijlage 2 verwijst u naar bijlage 1 voor een beschrijving van de procedures. De bijlagen dienen echter op zichzelf leesbaar te zijn. Kunt u daarom de betreffende teksten ook in bijlage 2 opnemen?

Zonder deze aanvullende informatie kan de beslissing nadelig voor u uitvallen

omdat de gegevens onvolledig of onduidelijk zijn.

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen 14 dagen na de datum van dit bericht op. U kunt dit aanleveren via het formulier op mijn.rvo.nl/dierproeven.

Uw oorspronkelijke aanvraag bevatte 3 bijlages, in de herschreven aanvraag heeft u een 4e bijlage toegevoegd. Hiervoor zult u een aanvullende factuur van ons krijgen.
Uw aanvraag zal worden besproken in de CCD vergadering van 31 oktober. Antwoorden die uiterlijk 30 oktober worden ingediend zullen worden meegenomen in de bespreking van uw aanvraag.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen? Kijk dan op www.centralecommissiedierproeven.nl voor meer informatie. U kunt ook een e-mail sturen naar info@zbo-ccd.nl. Of bel ons op: 088 042 1000.

Met vriendelijke groet,

Namens de Centrale Commissie Dierproeven

[redacted]

Dit bericht is automatisch gegenereerd en bevat daarom geen handtekening.

Centrale Commissie Dierproeven

Datum
17 oktober 2025

Onze referentie
Aanvraagnummer
AVD11500202519049

Centrale Commissie Dierproeven
Postbus 93118
2509 AC 's-GRAVENHAGE

Datum 23 Oktober, 2025
Onderwerp Response brief CCD
Referentie AVD11500202519049

Center for Translational Immunology (CTI)

[redacted]

[redacted]

Visiting address:
Lundlaan 6
3584 EA Utrecht
The Netherlands

Geachte commissie leden,

In reactie op de gestelde vragen over onze aanvraag "Deciphering neuro-immune interactions and metabolism in pain regulation" met aanvraagnummer AVD11500202519049 geven wij hieronder antwoord op de door u gestelde opmerkingen en vragen.

Niet technische samenvatting
In de tekst "fate animals kept alive" geeft u aan dat dieren waarvan geen weefsels worden verzameld zoveel mogelijk worden hergebruikt. In de tabel "Fate of animals kept alive" blijkt echter niet dat dieren worden hergebruikt. Kunt u verduidelijken of dieren worden hergebruikt of niet?
Antwoord: *We verwachten 100 muizen en 100 ratten nodig te hebben (gedurende 5 jaar), voor het leren/oefenen van technieken. Hiervoor zullen wij zoveel mogelijk gebruik maken van op dat moment beschikbare surplus dieren. Anderzijds zullen we dieren ook aanbieden voor hergebruik. Gebaseerd op afgelopen jaren, verwachten we dat 5% van onze dieren hiervoor in aanmerking komen (~250 muizen, ~30 ratten). Dus in totaal komen we dan op ~350 muizen en ~130 ratten voor hergebruik. Deze aantallen hebben we toegevoegd aan de tabel in de NTS.*

Kunt u de verwijzing naar het centrum voor proefdiervrije biomedische translatie verwijderen, zodat de anonimiteit gewaarborgd kan worden?
Antwoord: *We hebben dit verwijderd.*

Kunt u de NTS indienen in het verplichte Excel format?
Antwoord: De Excel hebben we ingevuld en sturen we als bijlage mee.

Onduidelijkheden
In bijlage 1, onder F (ongerief) beschrijft u dat 100 muizen en 100 ratten gebruikt worden voor training en derhalve terminaal ongerief ondergaan. In de tekst hieronder beschrijft u echter dat voor de ratten 20% licht ongerief zal ervaren en 80% matig ongerief. Kunt u deze inconsistentie oplossen?

Antwoord: *Excuus, hier hebben we het geheel niet helder beschreven. De 100 ratten en 100 muizen voor training zal een deel terminaal ongerief ondergaan (bv voor intrathecale injecties), maar ook een deel zal gebuikt worden zonder anesthesie (bv intraplantar injecties). De referentie naar 80% matig ongerief en 20% licht ongerief (sham) is gekoppeld aan gebruik van de 480 geplande ratten voor TNT/groove model. De nieuwe tekst is nu als volgt: For the planned 480 rats for experiments, we will use the groove/TNT model which will give moderate discomfort. Sham-operated animals will have mild discomfort.*

Tabel 5 in bijlage 1 is onduidelijk. Kunt u aangeven wat de grijze en zwarte vlakken betekenen?
Antwoord: *We hebben de legenda van de tabel uitgebreid met een tekst om aan te geven wat de grijze en zwarte vlakken betekenen. Deze tekst is toegevoegd: 'The light grey boxes indicate the selected "pain model" (mild or moderate), the mid-grey boxes are to indicate the selected procedure (one, two or three strategies), and dark-gray is the total expected cumulative discomfort.´*

In bijlage 2 verwijst u naar bijlage 1 voor een beschrijving van de procedures. De bijlagen dienen echter op zichzelf leesbaar te zijn.
Antwoord: *We hebben de procedures ook toegevoegd aan bijlage 2.*

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven

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Onze referentie
Aanvraagnummer
AVD11500202519049

Datum 7 november 2025
Betreft Aanhouden aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 11 juli 2025 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Deciphering neuro-immune interactions and metabolism in pain regulation". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD11500202519049.

In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Onduidelijkheden
U geeft aan dat de modellen die u toepast maximaal matig ongerief teweegbrengen bij de dieren. In bijlage VIII van de Richtlijn 2010/63/EU staat vermeld:
"Procedures waarbij de dieren waarschijnlijk een ernstige vorm van pijn, lijden of angst, dan wel langdurig een matige vorm van pijn, lijden of angst zullen ondervinden en procedures die waarschijnlijk ernstige hinder voor het welzijn of de algemene toestand van de dieren zullen opleveren, worden ingedeeld als „ernstig”."
Kunt u aangeven hoe u ervoor zorgt dat cumulatief ernstig ongerief wordt uitgesloten, ook als gevolg van een matige vorm van pijn die langdurig aanhoudt?

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

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen 14 dagen na de datum van dit bericht op. U kunt dit aanleveren via het formulier op mijn.rvo.nl/dierproeven.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen? Kijk dan op www.centralecommissiedierproeven.nl voor meer informatie. U kunt ook een e-mail sturen naar info@zbo-ccd.nl. Of bel ons op: 088 042 1000.

Met vriendelijke groet,

Namens de Centrale Commissie Dierproeven

[redacted]

Dit bericht is automatisch gegenereerd en bevat daarom geen handtekening.

Centrale Commissie Dierproeven

Datum
7 november 2025

Onze referentie
Aanvraagnummer
AVD11500202519049

Datum	4 December, 2025
Onderwerp	Response brief CCD
Referentie	AVD11500202519049 & AVD11500202519050

Geachte commissie leden,

Voor onze beide aanvragen AVD11500202519049 & AVD11500202519050 ontvingen wij dezelfde opmerking;

"In de ogen van de CCD is echter geen antwoord gegeven op de vraag of er een langdurige vorm van matig ongerief optreedt waardoor er volgens de definitie van bijlage VIII van de Richtlijn 2010/63/EU ernstig ongerief ontstaat. De CCD is van oordeel dat de modellen die gepaard gaan met een langdurige matige vorm van pijn geclassificeerd dienen te worden als ernstig. Dit betreft (een substantieel deel van) de modellen beschreven in bijlage 1. De CCD is voornemens uw aanvraag te vergunnen mits u het ongerief van de modellen beschreven in bijlage 1 classificeert als ernstig. De DEC zal tevens worden gevraagd een nieuwe ethische afweging te maken over dit verhoogde ongerief"

Center for Translational Immunology (CTI)

[redacted]

Visiting address:
Lundlaan 6
3584 EA Utrecht
The Netherlands

Antwoord:

Hoewel wij van mening verschillen over de inschatting van het cumulatief ongerief volgen wij uw verzoek en hebben alle modellen met matig ongerief en die langer dan 4 weken duren, in bijlage 1 geclassificeerd als **ernstig**. Daarnaast heeft de IvD aangegeven extra audits uit te voeren om het werkelijke ongerief in te schatten en dit correct terug te rapporteren aan de CCD.

Ondanks dat wij uw wens respecteren, willen wij toelichten hoe wij tot onze oorspronkelijke inschatting van **matig ongerief** zijn gekomen. Op basis van literatuur en eerdere ervaringen zien wij in de chronische pijnmodellen geen afwijkingen geven in activiteit, eet- en drinkgedrag, sociaal gedrag, vachtconditie of ademhaling. Veel van deze modellen zijn bovendien 'klassieke' modellen voor bijvoorbeeld artrose of langdurige ontsteking, die ook door andere onderzoekers worden gebruikt zonder dat deze als ernstig ongerief worden geclassificeerd.

Wij hebben uitgebreid overleg gehad met de IvD en collega's over de classificatie van cumulatief ongerief bij 'chronische' pijnmodellen. De kern van de discussie is dat de term *"langdurig"* niet eenduidig is gedefinieerd: gaat het om 2 weken, 1 maand, 3 maanden of langer? Deze interpretatie is cruciaal voor de classificatie van cumulatief ongerief en voor het differentiëren tussen modellen die significant in duur verschillen. Onze inschatting is mede gebaseerd op menselijke ethische normen. Vanuit het gelijkheidsprincipe geldt volgens ons, en in overleg met clinici: patiënten met

neuropathische pijn of artrose ervaren vaak maanden tot jaren matige pijn, maar dit wordt niet als cumulatief ernstig lijden beschouwd. Het onderscheid ligt in de continuïteit: patiënten ervaren geen constante, onbeheersbare pijn. Matige pijn over langere tijd kan belastend zijn, maar wordt niet gelijkgesteld aan ernstig lijden. Onze modellen weerspiegelen deze situatie: dieren hebben geen continue spontane pijn, maar kunnen kortdurende pijn ervaren, bijvoorbeeld bij specifieke stimulus/meting (zoals de Von Frey-test). Gedrag, eetlust, gewicht en sociale interactie blijven intact, wat suggereert dat er geen ernstig ongerief is.

Het voorbeeldscoresysteem van de FELASA/ECLAM/ESLAV-werkgroep, zoals opgenomen in het NCad-advies, ondersteunt dit standpunt (Zienswijze: Cumulatief Ongerief | Nationaal Comité advies dierproevenbeleid). In figuur 3 wordt een voorbeeld-scoresysteem voor neuropathische pijn gegeven, een van de modellen in bijlage 1 waar wij het hoogste ongerief verwachten. Dit model (duur minimaal 3 a 4 weken) wordt als **matig** geclassificeerd, mits de totaalscore op parameters zoals gewichtsverlies, vachtverzorging, ademhaling, voedsel- en waterinname, gedrag, wondgenezing en toestand van de geopereerde poot niet boven 5 uitkomt. Onze modellen scoren nooit hoger dan 2 op deze individuele parameters, dan wel hoger 5 voor de cumulatieve score.

Op basis hiervan is onze voorafgaande inschatting dat het ongerief **matig** en niet **ernstig** is voor deze langdurige modellen. Bovendien zijn de HEP-criteria zo opgesteld dat ernstig ongerief wordt voorkomen. Wij hopen hiermee meer inzicht te geven in onze oorspronkelijke beoordeling.

Desalniettemin hebben wij, zoals hierboven aangegeven, in bijgevoegde aanpassing van de aanvraag derhalve het risico op ernstig ongerief voor Bijlage 1 op 30% (AVD11500202519049) & 50% (AVD11500202519050) gezet. Dit is voor alle modellen die langer duren dan 4 weken (gebaseerd op onder andere het FELASA scoresysteem).

Met vriendelijke groet,

[redacted]

Centrale Commissie Dierproeven

> Retouradres Postbus 93118 2509 AC Den Haag

Universitair Medisch Centrum Utrecht
[redacted]
Postbus 85500
3508 GA UTRECHT

Centrale Commissie Dierproeven

Postbus 93118
2509 AC Den Haag

centralecommissiedierproeven.nl
088 042 1000
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD11500202519049

Datum 24 november 2025
Betreft Aanhouden aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 11 juli 2025 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Deciphering neuro-immune interactions and metabolism in pain regulation". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD11500202519049.

In uw aanvraag zitten voor ons nog enkele onduidelijkheden. In dit bericht leest u wat wij nog nodig hebben en wanneer u een beslissing kunt verwachten.

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

Onduidelijkheden
Uw aanvraag is vrijdag 21 november opnieuw behandeld in de CCD vergadering. Hierbij heeft de CCD uw antwoord op de gestelde vraag over het ongerief van de door u gebruikte modellen besproken. U geeft in uw antwoord aan dat u geen tekenen ziet van acuut ernstig ongerief. In de ogen van de CCD is echter geen antwoord gegeven op de vraag of er een langdurige vorm van matig ongerief optreedt waardoor er volgens de definitie van bijlage VIII van de Richtlijn 2010/63/EU ernstig ongerief ontstaat. De CCD is van oordeel dat de modellen die gepaard gaan met een langdurige matige vorm van pijn geclassificeerd dienen te worden als ernstig. Dit betreft (een substantieel deel van) de modellen beschreven in bijlage 1. De CCD is voornemens uw aanvraag te vergunnen mits u het ongerief van de modellen beschreven in bijlage 1 classificeert als ernstig. De DEC zal tevens worden gevraagd een nieuwe ethische afweging te maken over dit verhoogde ongerief.

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

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen 14 dagen na de datum van dit bericht op. U kunt dit aanleveren via het formulier op mijn.rvo.nl/dierproeven.

Wanneer een beslissing
De behandeling van uw aanvraag wordt opgeschort tot het moment dat wij de aanvullende informatie hebben ontvangen. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Meer informatie
Heeft u vragen? Kijk dan op www.centralecommissiedierproeven.nl voor meer informatie. U kunt ook een e-mail sturen naar info@zbo-ccd.nl. Of bel ons op: 088 042 1000.

Met vriendelijke groet,

Namens de Centrale Commissie Dierproeven

[redacted]

Dit bericht is automatisch gegenereerd en bevat daarom geen handtekening.

Centrale Commissie Dierproeven

Datum
24 november 2025

Onze referentie
Aanvraagnummer
AVD11500202519049

Centrale Commissie Dierproeven

Centrale Commissie Dierproeven
Postbus 93116
2509 AC Den Haag
centralecommissiedierproeven.nl
088 042 1000
info@zbo-cxd.nl

Onze referentie
Aanvraagnummer
AVD11500202519049

Bijlagen
3

Universitair Medisch Centrum Utrecht
[redacted]
Postbus 85500
3508GA UTRECHT

Datum : 08-12-2025
Betreft : Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted]

Op 11 juli 2025 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Deciphering neuro-immune interactions and metabolism in pain regulation" met aanvraagnummer AVD11500202519049. Wij hebben uw aanvraag beoordeeld.

Beslissing

Wij keuren uw aanvraag goed. Uit artikel 10a, eerste lid van de Wet op de dierproeven (hierna: de wet) volgt daarom dat het is toegestaan om de in de vergunning genoemde dierproeven uit te voeren binnen de gestelde vergunningsperiode. Deze vergunning wordt afgegeven voor de periode van 8 december 2025 tot en met 31 oktober 2030.

Aan de vergunning hebben wij de volgende voorwaarde verbonden op grond van artikel 10a1, tweede lid van de wet.

Beoordeling achteraf
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk oktober 2031 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

De onderbouwing van deze beslissing vindt u onder 'Overwegingen'.

Procedure

Advies dierexperimentencommissie
Wij hebben advies gevraagd bij de dierexperimentencommissie DEC Utrecht (hierna: DEC). Dit advies is ontvangen op 9 oktober 2025.
Wij hebben de DEC om aanvullend advies gevraagd over het ongerief.
Op 4 december 2025 hebben wij het aanvullend advies van de DEC ontvangen.

Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, derde lid van de wet.

Centrale Commissie Dierproeven

Datum
08-12-2025

Onze referentie
AVD11500202519049

Nadere vragen aanvrager
Op 17 oktober 2025, 7 november 2025, 24 november 2025 hebben wij u om aanvullingen gevraagd. U heeft tijdig antwoord gegeven.
De aanvullingen hadden betrekking op de beschrijving van de procedures, het ongerief en de Niet-Technische Samenvatting.
Uw reactie is betrokken bij de behandeling van uw aanvraag.

Overwegingen

Wij kunnen ons vinden in de inhoud van het advies van de DEC, inclusief de daaraan ten grondslag liggende motivering.

Beoordeling achteraf
Na afloop van het project moet er een beoordeling plaatsvinden zoals bedoeld in artikel 10a1, eerste lid, onder d en artikel 10a1, derde lid van de wet. De reden van deze beoordeling achteraf is dat in dit project dieren ernstig ongerief ondergaan. Deze beoordeling zal uiterlijk oktober 2031 plaatsvinden. Meer informatie over de eisen die gesteld worden bij de beoordeling achteraf vindt u in de bijlage 'Weergave wet- en regelgeving'.

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 93118, 2509 AC 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. Nadat u een bezwaarschrift heeft ingediend kunt u een voorlopige voorziening vragen bij de voorzieningenrechter van de rechtbank in de vestigingsplaats van de vergunninghouder. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisende situatie.

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 vergunninghouder valt.

Meer informatie

Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl, stuur een e-mail naar info@zbo-ccd.nl of neem telefonisch contact met ons op: 088-0421000.

Centrale Commissie Dierproeven
namens deze:

Bijlagen:
- Projectvergunning
- DEC-advies
- Weergave wet- en regelgeving

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven
Verleent de Centrale Commissie Dierproeven aan

Naam instelling of organisatie:	Universitair Medisch Centrum Utrecht
Straat en huisnummer:	Postbus 85500
Postcode en plaats:	3508GA UTRECHT
Deelnemersnummer NVWA:	11500

deze projectvergunning voor het tijdvak 8 december 2025 tot en met 31 oktober 2030, voor het project "Deciphering neuro-immune interactions and metabolism in pain regulation" met aanvraagnummer AVD11500202519049, na advies van dierexperimentencommissie DEC Utrecht. De functie van de verantwoordelijk onderzoeker is [redacted]

Het besluit is gebaseerd op de volgende (aangepaste) stukken:
1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 11 juli 2025
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen op 9 oktober 2025;
b Bijlagen dierproeven
- 3.4.3.1. Chronic pain models & measurements, zoals ontvangen op 5 december 2025;
- 3.4.3.2. Transient pain models & measurements, zoals ontvangen op 24 oktober 2025;
- 3.4.3.3. Obtaining donor cells & cells for ex vivo analysis, zoals ontvangen op 9 oktober 2025;
- 3.4.3.4. Breeding of genetically altered mice with discomfort profiles, zoals ontvangen op 9 oktober 2025;

c Niet-technische Samenvatting van het project, zoals ontvangen op 5 december 2025;
d Advies van dierexperimentencommissie, zoals ontvangen op 9 oktober 2025.
e De aanvullingen op uw aanvraag, zoals ontvangen op 24 oktober 2025, 13 november 2025, 5 december 2025

Naam proef	Diersoort / Stam	Aantal dieren	Ongerief	Overige opmerkingen
3.4.3.1. Chronic pain models & measurements				
	Muizen (Mus musculus)	2980	16,7% Licht 56,0% Matig 24,0% Ernstig 3,3% Terminaal	
	Ratten (Rattus norvegicus)	580	17,2% Licht 16,6% Matig 66,2% Ernstig	
3.4.3.2. Transient pain models & measurements				
	Muizen (Mus musculus)	1440	50,0% Licht 50,0% Matig	
3.4.3.3. Obtaining donor cells & cells for ex vivo analysis				
	Muizen (Mus musculus)	260	90,0% Licht 10,0% Terminaal	

	Ratten (Rattus norvegicus)	26	100,0% Licht	
3.4.3.4. Breeding of genetically altered mice with discomfort profiles				
	Muizen (Mus musculus)	550	100,0% Licht	

Voorwaarden

Beoordeling achteraf
In dit project worden dierproeven toegepast die vallen in de categorie ernstig volgens artikel 10b van de wet. Daarom bent u verplicht om na afloop van de vergunning in een Beoordeling achteraf over uw project te rapporteren. Deze beoordeling zal uiterlijk oktober 2031 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

Geldende voorschriften
Wij wijzen u op onderstaande geldende voorschriften, die volgen uit artikel 1d, vierde lid, artikel 10, eerste lid en/of artikel 10a3 van de wet.

- Go/ no go momenten worden voor aanvang van elk experiment afgestemd met de IvD.
- Het is verboden 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.
- Het is verboden dierproeven te verrichten voor een doel waarvan het belang niet opweegt tegen het ongerief dat aan het proefdier wordt berokkend.
- Overige wettelijke bepalingen blijven van kracht.

Aanvraagnummer:
AVD11500202519049

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, derde lid van de wet. Uit artikel 10b, eerste lid van de wet 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, eerste lid van de wet de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven. Artikel 10b, tweede en derde lid van de wet schrijven voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 van de wet 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.

Aanvraagnummer:
AVD11500202519049

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 van de wet 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 van de wet 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 van de wet 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.