	Inventaris Wob-verzoek W23-03									
		wordt verstrekt				**weigeringsgronden**				
nr.	**document NTS 202215867**	**reeds openbaar**	**niet**	**geheel**	**deels**	**5.1, lid 1c**	**5.1, lid 2e**	**5.1, lid 2f**	**5.1, lid 2h**	**5.2, lid 1**
1	Aanvraag projectvergunning, d.d. 17-02-2022				x		x		x	
2	Projectvoorstel bij aanvraag				x				x	
3	Bijlage dierproeven 1 bij aanvraag				x				x	
4	Bijlage dierproeven 2 bij aanvraag				x				x	
5	Bijlage dierproeven 3 bij aanvraag				x				x	
6	NTS bij aanvraag bij aanvraag				x				x	
7	E-mail CCD aan DEC verzoek om advies, d.d. 17-02-2022				x		x		x	
8	Brief aanpassing aanvraag projectvoorstel, d.d. 22-03-2022				x		x		x	
9	Aanvraag projectvergunning met aanpassing, d.d. 22-03-2022				x		x		x	
10	DEC advies, d.d. 30-05-2022				x		x		x	
11	Projectvoorstel na DEC advies				x				x	
12	Bijlage dierproeven_1 na DEC advies				x				x	
13	Bijlage dierproeven_2 na DEC advies				x				x	
14	Bijlage dierproeven _3 na DEC advies				x				x	
15	NTS na DEC advies				x				x	
16	Adviesnota aan CCD, d.d. 31-05-2022_met opmerkingen				x		x		x	x
17	Adviesnota aan CCD, d.d. 31-05-2022				x		x		x	x
18	E-mail CCD aan vergunninghouder over aanvraag projectaanvraag, d.d. 31-05-2022				x		x		x	
19	Bijlage dierproeven_2 na CCD vragen				x				x	
20	NTS na CCD vragen en definitieve versie			x						
21	Adviesnota aan CCD, d.d. 04-07-2022				x		x		x	x
22	Beschikking d.d. 05-07-2022				x		x		x	
23	E-mail CCD aan DEC, terugkoppeling over projectvergunning , d.d. 29-07-2022				x		x		x	

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*	5.1 lid2h
☐ 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	5.1 lid2h			
Titel, voorletters en achternaam van de portefeuillehouder	Titel	Voorletters	Achternaam	☒ Dhr. ☐ Mw
	5.1 lid2e			
E-mailadres contactpersoon	5.1 lid2e			
Titel, voorletters en achternaam van de diens gemachtigde (indien van toepassing)	Titel	Voorletters	Achternaam	☐ Dhr. ☐ Mw
E-mailadres gemachtigde				

Vul de gegevens van het postadres in.

Straat en huisnummer	5.1 lid2h
Postcode en plaats	
Postbus, postcode en plaats	

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	5.1 lid2e	☒ Dhr. ☐ Mw.
Functie	5.1 lid2e	
Afdeling	5.1 lid2h	

	Telefoonnummer	5.1 lid2e	
	E-mailadres	5.1 lid2e	
1.5 *(Indien van toepassing)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	(Titel) Naam en voorletters	5.1 lid2e	☐ Dhr. ☒ Mw.
	Functie	5.1 lid2e	
	Afdeling	5.1 lid2h	
	Telefoonnummer	5.1 lid2e	
	E-mailadres		
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	5.1 lid2h	
	E-mailadres	5.1 lid2h	
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 hier onder 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 geen negatieve gevolgen kan hebben voor het dierenwelzijn?

☒ Nee > Ga verder met vraag 3

☐ Ja > Geef hier onder 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	01-03-2022
	Einddatum (t/m)	01-03-2027
3.2 Wat is de titel van het project?	The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease	
3.3 Wat is de titel van de niet-technische samenvatting?	Communicatie tussen het enterisch zenuwstelsel, darm lumen en andere weefselomgevingen: van homeostase tot de ontwikkeling en progressie van verscheidene ziektes.	
3.4	Naam DEC	5.1 lid2h
	Postadres	

Wat is de naam van de Dierexperimentencommissie (DEC) van voorkeur?	E-mailadres	5.1 lid2h	

4 Factuurgegevens

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

Naam:		Afdeling:
Straat:		Huisnummer:
Postcode:	Plaats:	
Postbus:	Postcode:	Plaats:
E-mail:		

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

Ordernummer:

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	5.1 lid2e
Functie	
Plaats	5.1 lid2h
Datum	17 - 02- 2022
Handtekening	5.1 lid2e

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
1.3	Provide the title of the project.	The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease

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.

The **gastrointestinal tract** is built from a complex set of tissue components and cellular networks of distinct embryological origin that integrate their activity to continuously control gut function. This structure extends from the oral cavity to the rectum and performs essential roles such as digestion and absorption of nutrients and water, motility, and host defence to maintain health and homeostasis of organisms. Because of its position at the interface between the external and internal milieu of the body, it is not surprising that the gastrointestinal tract can be a possible entry point for microbes that have been recently recognised as major contributors in host physiology (1), but also in the pathogenesis of several diseases.

Major components GI-tract

The intricate anatomy of the gastrointestinal tract includes a mucosal layer, in which epithelial cells function as a physical barrier to a highly dynamic and complex luminal environment (e.g. microbiota), and work together with the largest collection of immune cells within the body (intestinal innate and adaptive immune systems) to establish a balance between host defence (e.g. pathogens, toxins) and tolerance (e.g. nutrients, commensal microbiota). Also, the gastrointestinal tract contains an extensive local vasculature, which is essential for nutrient absorption, and acts as a gateway for endocrine signals that are produced within the gut, and ensures communication with remote sites in the body. In addition, smooth muscle layers and Interstitial Cells of Cajal (ICCs) execute distinct programs to initiate and maintain intestinal motility, which is necessary for food propulsion, digestion and waste expulsion. We firmly believe that the ***enteric nervous system (ENS)***, the system of interest of our lab, works as the central hub that tunes this multifaceted set of chores to maintain gastrointestinal homeostasis (2).

The Enteric Nervous System: development, organization and functions

In mammals, the ENS is derived from neural crest cells that migrate throughout and colonise the entire length of the gastrointestinal tract. By receiving adequate inputs, ENS progenitors also migrate centripetally from the muscle layers towards the mucosa, colonising and differentiating into more mature and functional enteric glial cells (EGCs) and neurons that orchestrate most aspects of gastrointestinal function, including mucosal absorption and secretion, intestinal blood circulation, host defence and motility (3, 4).

Given the critical steering of ENS functioning to maintain gastrointestinal homeostasis, it is not surprising that developmental (i.e. congenital) or environmental (i.e. acquired) deficits in the ENS have been linked with severe neuropathies such as Hirschsprung's disease, and functional gastrointestinal disorders such as irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD) (5). In addition, the role of nerves in (e.g. colorectal) cancer is an emerging research field, as neurotrophic factors derived from enteric neurons have been shown to act in the development and progression of several cancer types (e.g. stomach) (6). In this context, we recently uncovered a role for the NDRG4 gene, one of the most accurate biomarkers for colorectal cancer (CRC) (7) which is specifically expressed in the ENS (8), in both ENS development and colorectal pathogenesis. In fact, ENS development and intestinal functioning is negatively affected upon knockdown of *ndrg4* in zebrafish (9) and lack of *Ndrg4* in mice alters enteric neuronal signalling and aggravates the progression of CRC (10). Furthermore, both published **(Figure 1)** and unpublished data from our lab, suggest a role for the ENS in the biology of CRC and that these cells can affect/communicate with each other (8, 10) which has to be studied further. Based on this, cancer therapy might have different effects when the ENS is altered, which is also an area of interest for our lab.

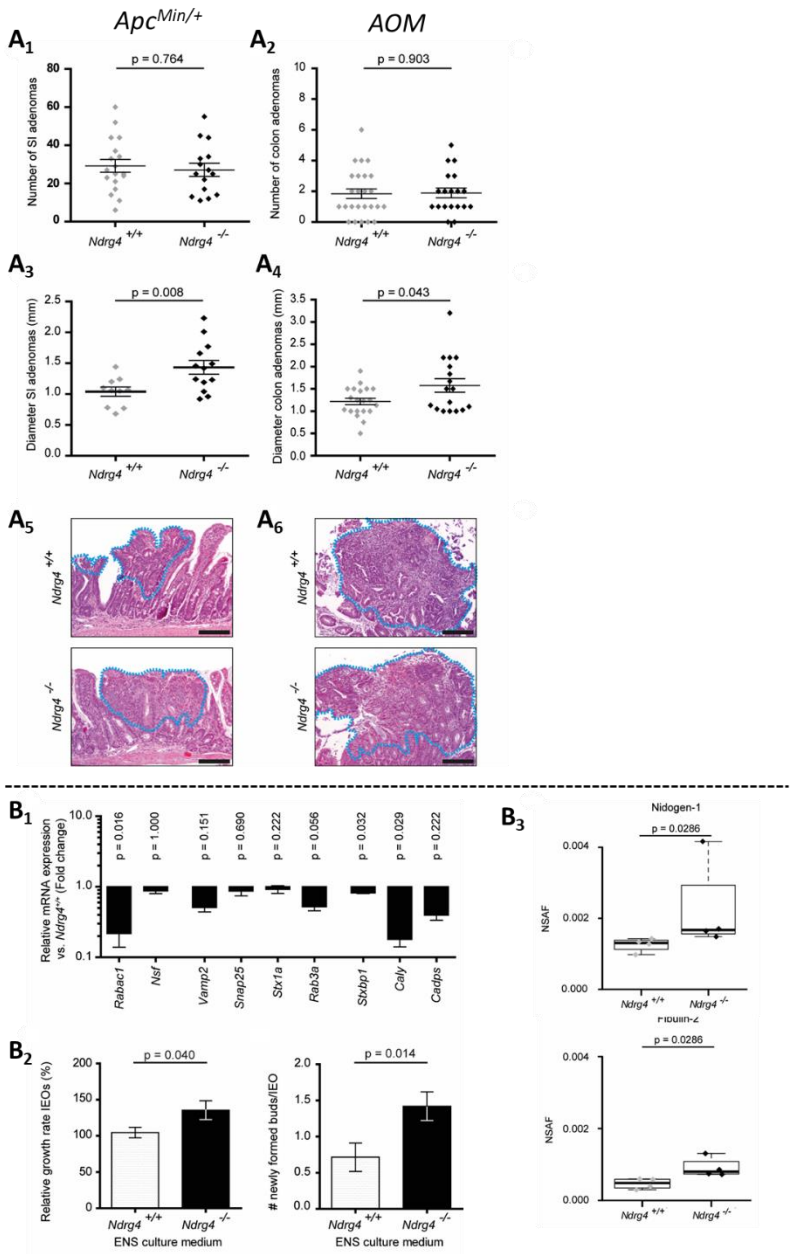

Figure 1: *Loss of enteric neuronal Ndrg4 is associated with enhanced CRC progression, most likely via affecting vesicle trafficking and the increased release of two extracellular matrix proteins (Nidogen-1 & Fibulin-2).* **(A)** Whereas loss of *Ndrg4* has no influence on the incidence of small or colonic adenomas **(A_1-A_2)**, it correlates with enlarged **(A_3-A_4)** and more aggressive **(A_5-A_6)** adenomas. **(B)** Mechanistically, primary ENS cultures characterized by loss of *Ndrg4* have revealed alterations in the expression of genes involved in vesicle trafficking **(B_1)** and medium derived from these cultures significantly enhances the growth of intestinal epithelial organoids **(B_2)**. Mass spectrometry on this culture medium uncovered the increased levels of two extracellular matrix molecules Nidogen-1 and Fibulin-2 **(B_3)**.

Although the ENS was first described many decades ago and considerable progress has been achieved in recent years understanding this system, there are still several challenges to overcome in mammals. Novel insights emerging from the transcriptional profile and organisation of enteric neuronglia units have shed light on the developmental principles that govern the assembly of the ENS (3, 11, 12). However, despite many similarities in the sophistication of neural circuits with other parts of the nervous system in vertebrates, the topographic organisation of the ENS cells within the gut wall is seemingly chaotic and very complex. Neuronglia units contain distinct neuronal and EGC subtypes that are arranged into two concentric and interconnected structures called plexuses, forming in a complex and heterogeneous wiring network within the intricate setting of the gut wall. In recent years, our and other laboratories worldwide have put an extensive effort to develop high-resolution techniques to identify and label ENS cells in order to map and understand the organisation of ENS components under physiologic and pathological conditions (1, 3, 11-17). Nevertheless, our understanding of **how ENS signalling arises from the mature neuroglial circuits to connect to intrinsic and extrinsic intestinal tissues (e.g. epithelial, immune cells, vasculature, microbiome and brain) lags far behind.**

The ENS partners with microbiota and the brain

The ENS functions partially independently of inputs from the central nervous system (CNS), yet it is a pivotal relay system in the bidirectional communication between the gastrointestinal tract and the brain (i.e., gut brain axis). The physiological importance of this microbiota-gut-brain axis has been highlighted by published work from our group and collaborators, revealing that tissue and behavioural defects on the ENS are associated with alterations in the microbial landscape (1). For instance, by combining genetic tools and novel strategies to label and isolate intestinal intrinsic neurons, we demonstrated in a collaboration with the Francis Crick Institute (London, UK) that microbiota regulate the physiology of the ENS. This study highlighted the consequences of tissue and environmental changes on ENS behaviour (from alterations in ENS transcriptomic landscape to the interruption of normal performance of tissue physiology) (1). Furthermore, in collaborative work with other groups at 5.1 lid2h , we have recently shown that maternal exposure to intestinal pathogens during pregnancy alters the levels of markers that are expressed exclusively by ENS cells in the foetus, and results in increased infiltration of immune cells (18). Altogether, work from our group and other (collaborative) laboratories proposes that the ENS is crucial in maintaining the normal physiology of the gastrointestinal tract by interacting with other tissues and systems. More importantly, these studies emphasize the plastic potential of the ENS as it responds to intrinsic (tissue) and extrinsic (environmental) cues that affect the gastrointestinal homeostasis. **However, the field still lacks on specific mechanisms by which ENS cells communicate with other systems to maintain tissue integrity or respond to insults.** Interestingly, we have recently found that EGCs upon helminthic infection with Heligmosomoides polygyrus (H. poly), significantly upregulate molecules that are involved in the production of extracellular vesicles (EVs; e.g., CD63, CD9) (5.1 lid2e, 5.1 lid2h

These data **suggest** a putative role for EGCs-derived EVs in enteric cell-to-cell communication e.g. by carrying epitranscriptomic modulators such as miRNAs (Holland et al., unpublished observations) in disease progression and/or resolution.

Consequently, our project **aims to** develop a holistic and advanced approach to study the influence of the intestinal environment on ENS transcriptome, morphology and physiology, both in health and disease. More specifically, we want to further **investigate how the ENS responds to physiological challenges in the luminal environment (e.g., microbiota) and to local pathologies (e.g., cancer and helminthic infections)**. At the same time, we will assess how alterations in ENS homeostasis (e.g. caused by microbial changes) influence other parts of the nervous system (e.g. central nervous system/ peripheral nervous system, as part of the gut-brain axis).

To achieve and succeed in this project, we are committed to apply well established *in vitro* analysis of ENS cell types and intestinal organoids to reduce animal use. We have confidence that all experiments performed under this project license will lead to a better understanding of the adult ENS. We believe that any progress in understanding the spatial organisation, the molecular landscape and the function of the ENS, as relevant as it will be, unravels only small fragments of the gut biology. Therefore, and to better comprehend the aetiology and the consequences of enteric neuropathies and other diseases that affect gastrointestinal function, we will have to apply integrative approaches that take into account the dynamic interactions between the ENS surrounding intestinal tissues and the luminal microenvironment.

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

The **ultimate goal of this project** is to elucidate the molecular pathways that orchestrate the organisation and function of the ENS, and its integration with other tissues (e.g., brain) and systems (e.g., immune, vascular and epithelial systems) during physiological challenges (e.g. microbiota) and pathological challenges (e.g. gastrointestinal diseases like cancer, worm infection). This will bring us closer to understand how the ENS participates in the onset and progression of diseases that affect the gastrointestinal tract, and therefore will eventually contribute to the development of new targets and therapies to tackle gastrointestinal disorders. For this purpose, we aim to develop a holistic and advanced approach to study the influence of the intestinal environment on ENS transcriptome, morphology and physiology, both in health and disease, for which we will use well-established murine models and procedures.

It is important to state that this project license will work as an umbrella to cover 4 parallel PhD projects in our group. Therefore, the main question in this project proposal will be minutely investigated by addressing 3 sub-aims (= immediate goals), which are described below.

Aim 1: Generate and use *in vitro* (co- culture) systems to study cell-to-cell communication (Also used to address aims 2, and 3) – Appendix 1

Because studying communication between different systems/cell types *in vivo* is difficult, we will generate and use *in vitro* systems to study mechanistical insights dictating cell-cell communication and use this information to increase the likelihood of success in aims 2 and 3 (e.g. by targeting/labelling/studying specific cells/vesicles/molecules that we found here in aim 1). We will assess potential communication mechanisms like EVs and miRNAs in (in-)direct co-culture models using different (primary) cell types like ENS cells versus CRC (co-cultures with available CRC cell lines), intestinal organoids, epithelial and/or endothelial cells (see Strategy section for detailed explanation and justification). Importantly, whenever possible, preliminary data will be acquired by using *in vitro* systems (both cell lines and primary cell cultures) prior to experiments that might cause long lasting harm, pain and distress to experimental animals *in vivo*.

Aim 2: Investigate cellular and molecular mechanisms of interaction between ENS, intestinal (cancer) and other cells in a cancerous environment – Appendix 2

Both published (Figure 1) and unpublished data from our lab, suggest a role for the ENS in the biology of CRC and that the ENS can affect/communicate with other intestinal cell types (8, 10). The CRC-related research described in this PL follows and elaborates on our findings and ideas obtained with the previous PL (AVD5.1 lid2h), as described in detail in appendix 2. With these experiments, we aim to further delineate the participation of EGCs and neurons on the onset and progression of CRC, the effects of their interactions on both CRC cells and ENS cells and the possible mechanisms involved in this interaction (see Strategy section for detailed explanation and justification). Further explanation of read-outs and techniques is described in the Strategy section and Appendix 2.

Aim 3: Investigate the mechanisms of cell-to-cell communication in homeostasis and diseases that affect the gastrointestinal tract (e.g., worm infection, (and/or) microbiota alteration, (and/or) inflammation)- Appendix 3

Preliminary data from our lab and collaborators suggests that the infection model with *H. poly* is ideally suited to address the research question in aim 3, as it affects gastrointestinal homeostasis and triggers an ENS response important for disease resolution. However, the latter needs further investigation. After acquisition and analysis of data using this model and depending on the outcome/mechanisms or cell types that seem to be involved, we will decide on the road to further investigate other gastrointestinal challenges (e.g., inflammation, microbiota manipulation) to confirm whether the ENS phenotype and involved molecular pathways observed in response to *H. Poly* are shared amongst other gastrointestinal diseases. Further specification of this and explanation of read-outs and techniques is described in the Strategy section and Appendix 3.

The experiments performed under this project license are aimed to dissect the pathways by which the ENS interacts with other cellular systems in health and disease. We will investigate e.g. the role of ENS cells, ENS-derived EVs and miRNAs in controlling gastrointestinal function, disease progression and resolution *in vitro* and *in vivo*.

3.2.2 Provide a justification for the project's feasibility.

All experiments suggested above can be successfully performed during the course of this project as the expertise and facilities needed to execute them are available, either in the host or in other laboratories, with whom we have established solid collaborations. In fact, the host lab has been able to establish optimized protocols for the successful generation of all primary cell types and (in-) direct co-culture models with the previous PL. Also, plenty of expertise regarding the different CRC and CRC-treatment models have been gained with the previous PL. Expertise regarding the other models have been gained by the collaborative labs, who are currently still advising and helping us with respect to these models.
Although it will be the dominant topic of interest of our laboratory, our work will not only include analysis on the ENS but will also represent contributions from other groups/institutes that have expertise on other non-ENS systems, interests, reagents and materials, wildtype or genetically manipulated animal models, to fully integrate the foundation of Science, and improve the impact, relevance and reproducibility of the experiments adhering the premises of the 3Rs.

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.

3.3 Relevance

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

Relevance for research: Research interest focusing on the gut has increased in recent years because of the clinical and biological relevance of this organ in several diseases. More specifically, it has been recently shown (by our and other groups) that the ENS interacts with multiple systems and has been implicated in the onset and progression of many diseases that not only affect the gastrointestinal tract but also the CNS (5). Due to the intricacy of its network and close proximity with many other tissues, studying the nervous system of the gut is challenging and requires expertise from distinct fields. Our group and collaborators have all necessary tools and skills to thoroughly dissect the cellular and molecular pathways that underlie the ENS crosstalk with other systems in various conditions. Given the similarities in composition, organisation and function between the ENS and the CNS, investigating the role of the ENS in homeostasis and disease would allow scientists to take the complexity of the gut to study other organs. We expect to unite different fields to unravel many other biological questions raised in this project and contribute for the consolidation of enteric neuroscience.

Relevance to the patient: Increasing evidence shows that the ENS might be the “entrance door" for several pathologies, including those affecting brain homeostasis. In neurodegenerative diseases, for instance, ENS phenotype and gastrointestinal malfunction have been shown to precede brain and/or motor symptoms by several years (5). Moreover, the ENS is likely to contribute to carcinogenesis, as both neo-neurogenesis and perineural invasion are unfavourable factors for CRC patients, which indicates that their survival rate is negatively affected by the higher nerve density in the tumour area (i.e. neo-neurogenesis) and invasion of tumour cells throughout nerve fibres (i.e. perineural invasion) (6). With confidence, it is conceivable to suggest that the participation of the ENS in diseases that affect the gastrointestinal tract and systems beyond it (i.e., gut-brain axis) deserves more attention.
We aim to study whether the ENS functions as a key player in the maintenance of the fitness of the intestinal microenvironment, and its crosstalk with the brain. Furthermore, we consider the ENS as a potential target for therapies in diseases that affect the homeostasis of the gastrointestinal tract in humans. Our prospect is thus to unravel the role of ENS behaviour in homeostasis and under circumstances that disturb the equilibrium of the healthy gastrointestinal tract. Therefore, we will include relevant animal and *in vitro* models to mimic human conditions, (e.g., cancer and helminthic infections), that impair intestinal function. By using advanced technology to study the ENS and powerful insights from experts in gastroenterology, we intend to identify novel cellular and molecular mechanisms, and biomarkers (e.g., EV-derived small molecules, non-coding miRNAs) that translate the onset of diseases progression and/or (response to) treatment.

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

The project stakeholders include the mice, the scientific community - in the same and related research areas- and ultimately patients, physicians and the pharmaceutical industry. The scientific community will immediately benefit from our findings. Exploring the basic mechanistics described within this PL allows us to identify new pathways and targets, which will ensure that our understanding of these diseases is improved. Besides, these fundamental insights might have implications for related diseases. Results will be published within peer-reviewed journals making them available to the entire scientific community. This ensures that our group will be identified as “experts” within this research field and allows other researchers to take advantage of these insights to further explore mechanisms and develop therapies. Long-term benefits are for patients, clinicians and health systems as our research will delineate new insights in the different disease mechanisms. Also, the pharmaceutical industry will benefit from this research project in case we find putative and novel cellular targets in diseases that alter the homeostasis of the GI tract. Finally, the mice represent the only stakeholders that do not immediately benefit from this project. Despite potential discomfort, these stakeholders are vital for this PL because the represented *in vivo studies* will allow us to gain new insights which will eventually ensure that all other stakeholders benefit from this project. On the other hand, we will try to minimize the need for *in vivo* studies with discomfort as much as possible by generating and using the *in vitro* models (aim 1).

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 study our main goal “the cell-to-cell communication between the ENS and intestinal and extra-intestinal tissues in health and disease” we have formulated the following specific aims and sub-aims:

1. **To generate and use *in vitro* systems to study cell-to-cell communication (Appendix 1),** we will derive cells from murine intestines and use these in *in vitro* analysis to assess the communication between the ENS and other cell types. These model systems will also allow studies of intracellular signalling mechanisms involved in intercellular communication and this information will be used to target/label/study for example specific cells/vesicles/molecules to address aims 2 and 3. More information on the numbers of animals/cell types and read-outs are described in Strategy below and appendix 1.
2. **To investigate cellular and molecular mechanisms of interaction between ENS, intestinal (cancer) and other cells in a cancerous environment (Appendix 2),** we will study the role of enteric neurons and EGCs in the onset and progression of CRC.
   Aim 2a: The role of EGCs in the development, progression, and treatment of CRC
   Aim 2b: The role of enteric neurons in the development, progression, and treatment of CRC
   More information on the numbers of animals and read-outs are described in Strategy below and appendix 2.
3. We will **investigate the mechanisms of cell-to-cell communication in homeostasis and diseases that affect the gastrointestinal tract (e.g., worm infection, (and/or) microbiota alteration, (and/or) inflammation) (Appendix 3)** to establish the role of ENS-derived molecules (e.g., EVs, miRNAs) to maintain the fitness of the gastrointestinal tract *in vivo*, and in the progression and resolution of diseases that affect the gut. The first model of choice is the *H. Poly* (worm infection). In this model we will study which phenotype will arise. Depending on whether dysregulation in the immune system, and/or the microbiome and/or ENS target genes arise, then animals can be subjected to microbiota alteration and/or inflammation (see Figure 4, Go/no-go moment).
   Aim 3a: The role of ENS-derived EVs for cell-to-cell communication in health and disease.
   Aim 3b: The role of ENS-derived miRNAs
   More information on the numbers of animals and read-outs are described in Strategy below and appendix 3.

To study the above-described aims and sub-aims, we will use wildtype mice and mice with altered ENS **(explained below in 3.4.2)** that are characterised by fluorescently labelled* (ENS) cells and are either unchallenged (i.e., non-diseased gut) or challenged with various stimuli to mimic or elicit certain diseased conditions (e.g., colorectal cancer, worm infection, alterations in intestinal microbiota…).

**Fluorescent labelling (including activity reporters such as genetically-encoded Ca^{2+} indicators) of the cells in the murine models will either be achieved by crossing mice that are (epi)genetically altered to drive expression in a cell-specific manner (based on promotor activity), with mice harbouring transgenes for conditional fluorescent reporter or by labelling cells by means of viral vector (e.g AAV) transduction.*

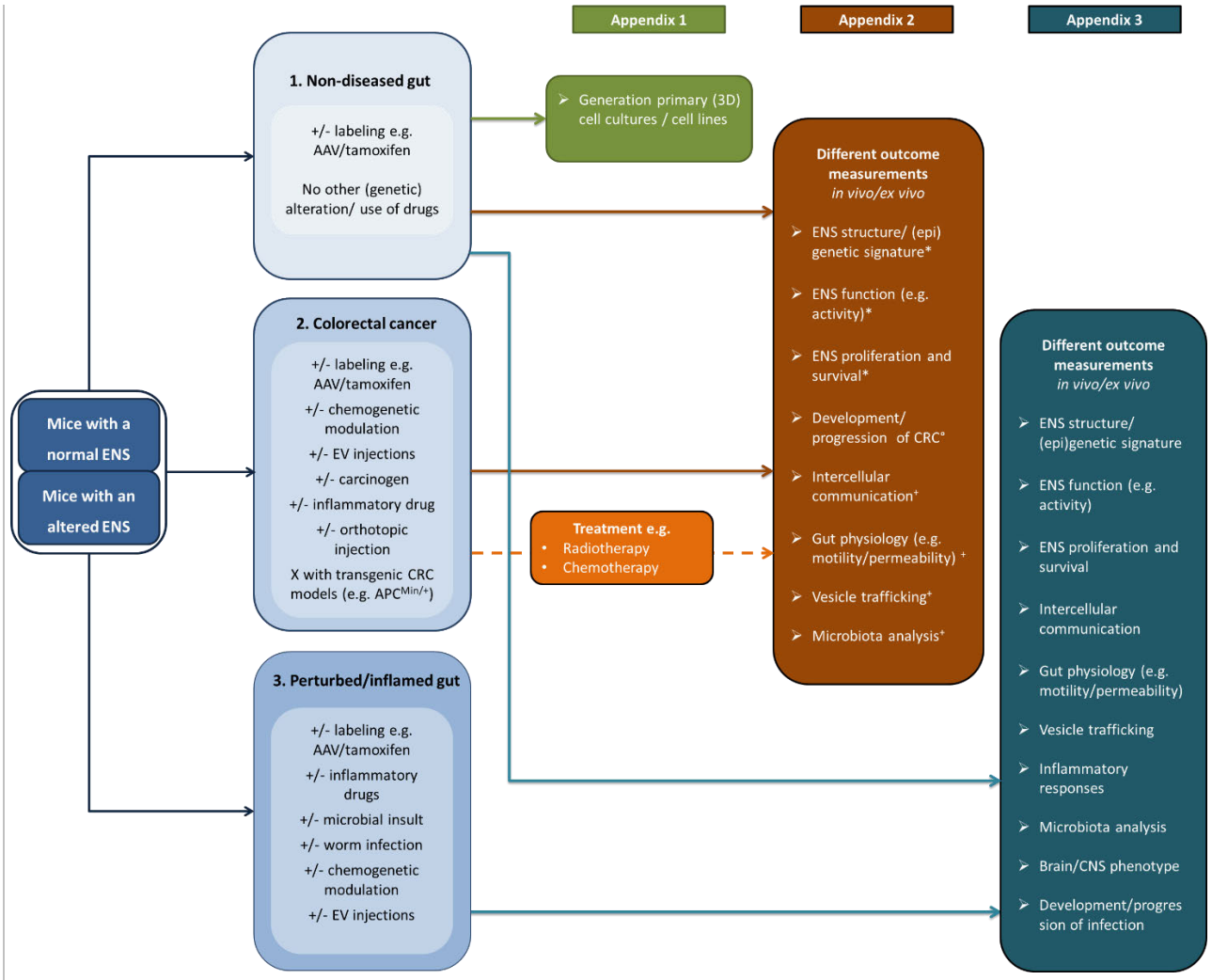

Figure 2. *Flowchart that will be followed over the duration of this project license, depicting the working model for the wild-type and transgenic murine models that will be used to examine the above-described goals. Note: *, +, ° behind the outcome measures of appendix 2 correspond to one of the green blocks in Figure 3 used to specify the strategy for this aim.*

In appendix 1 (Figure 2, green colour), we will generate and use cell culture models (e.g., ENS cells, neurospheres, 3D cultures) derived from the intestines of mice with a normal ENS and from mice with an impaired ENS to examine the communication between the ENS and other cell types *in vitro* (max 10 cell-cell interactions such as enteric neurons and enteric glia cells with macrophages, smooth muscle cells, epithelial cells, cancer cell lines) and intracellular signalling mechanisms involved in intercellular communication (max 7 signalling pathways such as calcium signalling, different extracellular vesicles (e.g. CD63+, miRNAs) (justification of these interactions and signalling pathways can be found within the background section: Major components GI-tract (p4) and The ENS,: development, organization and functions & The ENS partners with microbiota and the brain (P2-4), respectively). If available, established (secondary) cell lines will be used prior to any use of animals for culturing primary cells.

- **Animal models:** Wildtype mice, mice with labelled/activity modulated ENS cells and molecules, and mice with a (genetically) altered ENS;
- **Animal procedures:**

We will isolate murine gastrointestinal tracts from mice models with labelled cell types and/or an altered ENS (e.g. cell numbers, activity or miRNA expression) to generate homogeneous and mixed primary (3D) cultures (e.g., intestinal cells, EGCs, neurons and progenitors, and organoids). These different cell types will be used in *in vitro* experiments to study the interplay between the ENS and gut functioning in the non-diseased gut.

To investigate the role of ENS cells (e.g. vesicle secretion/ENS activity/miRNA) in the gut and their interactions with epithelial/immune/cancer cells (co-cultures with available cell lines), animals from the different mouse lines will be sacrificed after weaning for the generation of primary, mature (3D) cell cultures. In case of culturing progenitor cells and neurospheres, neonate mouse offspring will be used.
The animals may be subjected to administration of substances (e.g., tamoxifen, BrdU) and viral particles (e.g., xAAVs) to induce labelling (e.g. fluorescent reporter) or modulation of ENS cells and/or mark cellular processes (e.g. cell proliferation, cell death).
We will have to isolate all cell types from each model independently, because specific cell types need to be labelled and/or changes in the ENS need to be induced. Answers derived with either of these models may provide some useful insights that can be used/applied in the other models, but will definitely not provide answers we aim to address with the other models. Thus, given that all models serve their own purpose and will be used to answer specific questions, we cannot specify specific go/no-go moments within this aim.

In appendix 2 (Figure 2, orange colour), *in vivo* and *ex vivo outcomes* (listed below) will be investigated in our murine models of colorectal cancer compared to non-diseased gut and/or different genotypes that affect the ENS compared to each other in a CRC model. These CRC models are described in more detail in appendix 2. Below and in figure 2, we have defined 8 outcome measures, which will allow us to investigate the interplay between the ENS and CRC cells, and how they affect other cell types that are part of the tumor microenvironment, such as immune cells and microbiota, which will be studied along the ENS-CRC interaction (justification for these measures follows from the background section):

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)*
- ENS function (e.g., ENS connectivity and network activity)*
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)*
- Development/progression of CRC (e.g., track tumour burden)$^{+}$
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)°
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)°
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)°
- Microbiota analysis (e.g., composition and abundance, bacterial secretome)°

*Note: *, $^{+}$, ° correspond to one of the green blocks in Figure 3.*

- **Animal models:** Control mice and mice treated with carcinogenic/inflammatory agents or crossed with the APC$^{Min/+}$ mouse model and/or mice with labelled/activity modulated ENS cells or molecules and/or mice with altered ENS;
- **Animal procedures:**

Here, we aim to investigate and unravel the interface of the adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota, CNS) in health and colorectal cancer using well-established cancer induction protocols (e.g. AOM, AOM/DSS) or genetically induced cancer. Hereby, we will explore the interaction between the ENS and CRC and the possible mechanisms involved in this interaction based on the procedures/outcome measures described above (maximum of 8 outcome measures to study all interactions and mechanisms). In addition, after euthanization of our CRC mouse models, we will isolate tumours and adjacent normal epithelium, to generate intestinal (tumour) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact mechanism by which the ENS affects the epithelial and tumour cells and vice versa. In case of success in acquisition and analysis of data using these models (go/no-go), we will also study the potential impact of an impaired ENS (genetically/chemically-induced alteration) on current treatments of CRC (e.g. radio- and chemotherapy).

We will use these different mouse lines, CRC disease models and procedures to characterize the ENS and the interplay with CRC cells and other cell types that are involved in these processes.
Because this research is mainly set up to properly characterize the cell types, processes and pathways involved, it is difficult to clearly specify Go/No-go moments as this would limit the progress of the characterization process. However, in the figure below, we tried to clarify and structure our research strategy for this aim using decision moments where possible (**Figure 3**). Based on experience derived from the experiments in AVD5.1 lid2h we know that most of the disease models work sufficiently to induce CRC, so these models can be immediately applied to study all outcome measures in detail. To start with, the chemical cancer induction models: AOM and AOM/DSS are the models of choice (specifics about the models can be found in appendix 2). However, we want to use the genetic CRC APC model or an orthotopic CRC model to answer specific questions e.g. labelled cell tracking, mechanistic insights human CRC process. After analysis of the gained CRC data based on changes in cancer development/progression (outcome measure identified with $^{+}$ above),

we will know whether it is worthwhile (e.g. sufficient effects of the ENS on CRC process) to apply chemo/radio therapy (Go/No-go, Figure 3 left). After analysis of our outcome measures identified with a * above that study ENS structure, function, proliferation and/or activity, we will decide on the need (e.g. sufficient effect of CRC on the ENS) for further functional studies using the other outcome measures identified with a ° above (Go/No-go, Figure 3 right).

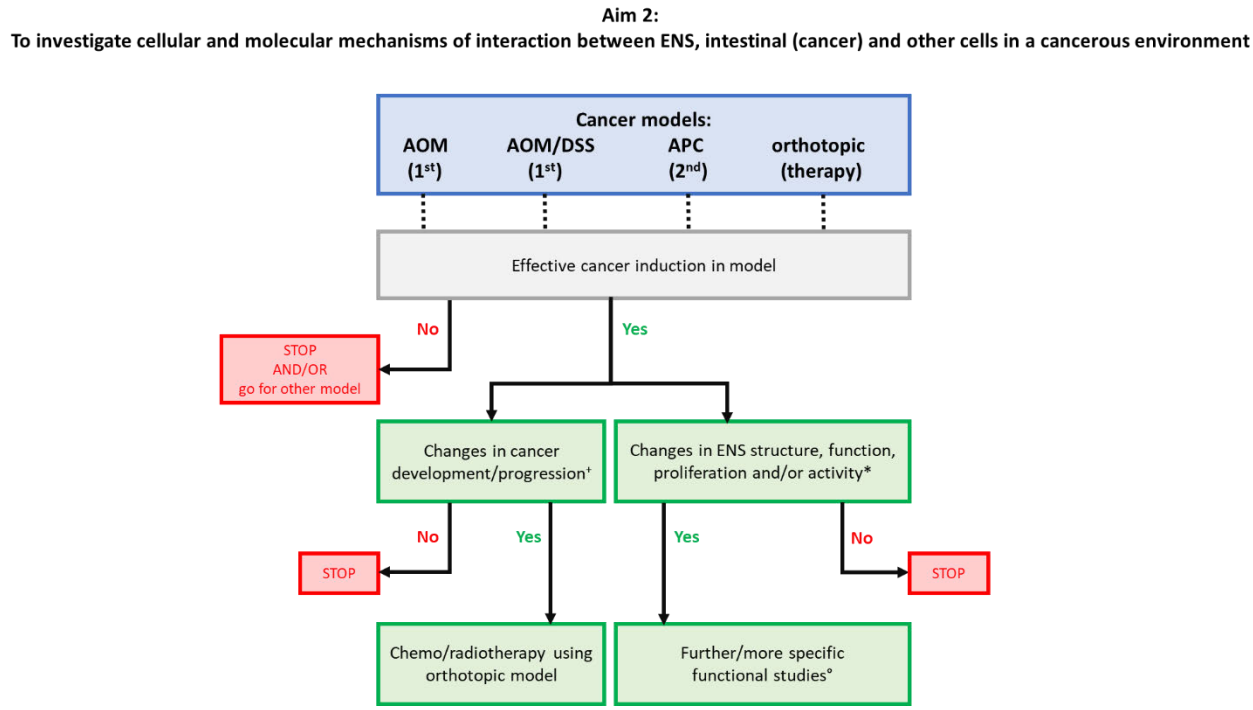

Figure 3. *Flowchart depicting crucial Go/No-go decision moments in appendix 2. *, +, ° in the green boxes correspond to the outcome measures described above and in Figure 2 (see above).*

Note 1 - Although experience gained with PL 2017-026 reveals that the different cancer models are efficient in several of the models that we will also use in this PL (e.g. Ndrg4, Hand2fl, NSE nogging) we are currently still unsure about the effectiveness in all other models that will be used in this PL. Also, given that we will have to re-order the APCmin/+ mice as we do not have them available within our facility anymore, we do not know if the efficiency of spontaneous cancer development will be similar as to what we have observed previously.
Note 2 – The "Further/more specific functional studies" are specified in detail within appendix 2 and entail the outcome measures identified with a + in the text above. Data derived from these experiments will provide further insights into the functional mechanisms that are of importance during cancer development and will enhance our understanding of colorectal pathogenesis.

In appendix 3 (Figure 3, dark blue colour), *in vivo* and *ex vivo outcomes* (listed below) will be investigated in our murine models of inflammation or other altering physiological cues compared to non-diseased gut (maximum 4 different models) and/or different genotypes that affect the ENS compared to each other in a disease setting (maximum 4 different genotypes for the 1st model and maximum 2 genotypes for follow-up models) (see information below and Figure 4). These models and the outcome measurements used for each model are specified in detail in appendix 3. To investigate the response of the ENS to physiological cues and inflammatory diseases that affect gastrointestinal homeostasis we will focus on maximum 10 different outcome measurements (further justification for these outcome measures follows from the background information):

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)
- ENS function (e.g., ENS connectivity and network activity)
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)

- Inflammatory processes (e.g., immune cell phenotype)
- Microbiota analysis (e.g., composition and abundance, bacteria secretome)
- Brain/CNS phenotype (e.g., organisation, non-invasive behavioural tests, imaging)
- Development/progression of infection (e.g., track parasitic infection and worm burden, numbers and eggs)

- **Animal models:** Wildtype mice and mice with labelled/activity modulated ENS cells or other molecules (control vs perturbed/inflamed gut).
- **Animal procedures:**

Here, we aim to investigate and unravel the interaction between adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota, CNS) in health (1) and/or local and systemic challenges, such as worm infection (H. poly), (epi)genetic editing (AAVx-carrying genetic modifiers), and/or intestinal inflammation (DSS or BAC), microbiota alterations (depletion/antibiotics), chemogenetic modulation (DREADDs) (further justification for these models follows from the background information). The same lines can be further examined by rescue experiments to re-establish the intestinal microbiota composition (reintroduction of microbes by faecal transplantation), rescue of specific phenotype by using advanced technology to deliver molecules (AAVx.transgene).

The *H. Poly* (worm infection) model will be first used to study this aim as it affects gastrointestinal homeostasis and triggers an ENS response important for disease resolution. If dysregulation in the immune system, and/or the microbiome and/or ENS target genes, arise from the worm infection challenge, then animals can be subjected to one or more of the follow-up challenges separately (e.g. intestinal inflammation challenge, genetic editing and alteration in intestinal microbiota challenge). These challenges can subsequently be used to study underlying mechanisms for the dysregulation of the systems/cells found in the *H. Poly* model (maximum 10 different outcome measures). Figure 4 below depicts the go/no-go strategy which is further described in detail in appendix 3.

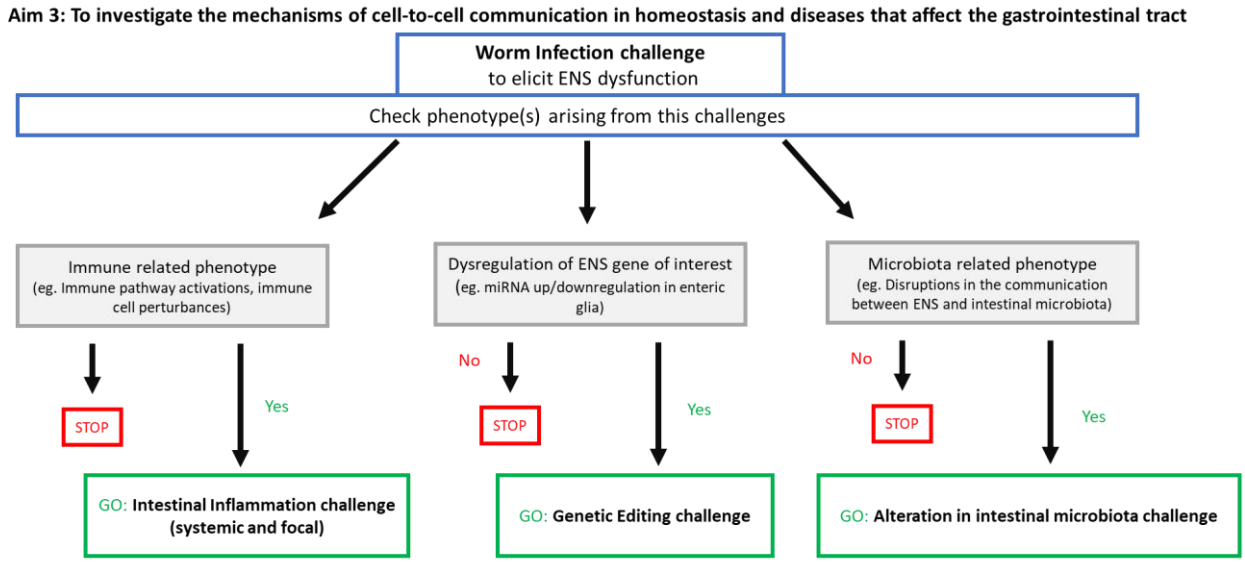

Figure 4. *Flowchart depicting crucial Go/No-go decision moments in appendix 3.*

ENS homeostasis will be analysed *in vitro* **(appendix 1),** *in vivo* and *ex vivo* **(appendix 2 and 3)** taking advantage of well-established animal models described in the respective appendices. To investigate the role of the ENS in homeostasis and disease, we will use mice with normal and impaired ENS that can be subjected to various challenges (e.g., induction of CRC, worm infection and alteration in intestinal microbiota). Outcome measures in aim 2 (appendix 2) and aim 3 (appendix 3) largely overlap as we want to study the effect/function of the ENS in both disease models (CRC vs inflammation/other perturbations). However, unique outcome measures that are specific for a disease model/perturbation are also implemented.

We will use *in vitro* assays using e.g. cell cultures whenever research questions (aim 1-3) can be addressed using these systems and prior to performing experiments in living animals if in vitro assays are available to study the outcome measure. When *in vivo* experiments are unavoidable, we will opt for procedures that cause the least pain, discomfort and distress and the shortest lasting harm.

3.4.2 Provide a justification for the strategy described above.

In this project, we will investigate how the ENS communicates with intestinal and extra-intestinal systems and its role in the onset and progression of disease. More specifically, in order to investigate how the ENS interacts with other systems, we will take advantage of well-established mouse models (e.g., cancer (10), inflammation (10), worm infection (5.1 lid2e, 5.1 lid2h *)*, microbiota alteration (1) and direct manipulation of the ENS itself (1) to unravel how it interacts with other cellular systems (ENS versus the immune and the vascular systems, versus the epithelial (cancer) cells, microbiota and the brain) in homeostasis and disease. To understand how one system functions, we need to acknowledge its interactions with the surrounding systems. These interactions support the idea that all progress achieved in any single tissue system, as valuable as it will be, only represents a proportion of the gut "tale", and that complete understanding of intestinal biology and gastrointestinal diseases will require integrative approaches that take into account the dynamic interactions between all intestinal tissues and the luminal microenvironment in health and disease. Hence, to have this integrative approach between the intrinsic nervous system of the gut and other non-ENS systems and identify putative mechanisms that dictate such complex gastrointestinal function in health and diseases, different animal models to label enteric nerve cells and manipulate the homeostasis of the gastrointestinal tract become essential to successfully address the enigmatic biology of ENS cells. The state-of-the-art models proposed here are necessary to 'model' the highlighted GI disturbances (e.g. CRC) and/or are needed as tools to investigate the involved molecular (e.g. miRNAs) and (patho) physiological (e.g. Ca^{2+} signalling) mechanisms.
For aim 1, all models serve their own purpose and will be used to answer specific questions. Therefore, we cannot specify specific go/no-go moments for this aim. For aim 2, we need to study different characteristics/processes/pathways that can be involved independently, however we tried to specify Go/No-go moments within out outcome measures. These outcome measures (8 categories in total) include ENS structure/ (epi)genetic signature, ENS function, ENS proliferation and survival, intercellular communication, gut physiology, vesicle trafficking, microbiota analysis and development/progression of CRC (see Strategy for more detailed explanation of these outcome measures and appendix 2 for further details). The outcome measures ENS structure/ (epi)genetic signature, ENS function, ENS proliferation and survival will be studied first, because the ENS is our system of interest (justification, see background). When changes in these outcome measures are observed in the context of cancer, further functional studies will be carried out regarding the outcome measures intercellular communication, gut physiology, vesicle trafficking, microbiota analysis to study mechanistical cues of the effect that was seen from the ENS-cancer interaction (justification for these outcome measures, see background). The outcome measure development/progression of CRC will also be studied immediately as this is our model system of interest in aim 2 (further justification, see background). After analysis of this data, we will decide on the possibility to use chemo/radio therapy if there is an effect of specific genetic models on cancer induction to see if they also respond different to cancer treatment. This will be a go/no-go moment as we will not start this study if there are no effects in the previous data (Figure 3). For aim 3, the *H. Poly* (worm infection) model will be used first and after analysis, we will decide which models will be used depending on the dysregulated system(s) found in the worm infection model. This will be a go/no-go moment and is also depicted in the figure above (Figure 4).

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	Creating and use of primary cell lines, organoids, neurospheres
2	Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer
3	Evaluating the crosstalk between the ENS and other systems on homeastasis and the onset and progression of inflammatory gut diseases
4	
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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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: Creation and use of primary cell lines, organoids, neurospheres

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.

As depicted in figure 1 (green box), the primary outcome parameter of this appendix is the generation of homogeneous and mixed primary (3D) cultures, which we subsequently use to study cell type characteristics (e.g. morphology, proliferation, migration), activity, and cross-talk between multiple cell types.
For this purpose, we will isolate murine gastrointestinal tracts from mice with labelled cell types and/or an altered/modulated ENS (e.g. cell numbers, activity or miRNA expression; details models described below the figure) to generate homogeneous and mixed primary (3D) cultures (e.g., intestinal cells, enteric glia cells (EGCs), neurons and progenitors, and organoids). These different cell types will consequently be used in *in vitro* (co-)culture experiments to study the interplay between the ENS and gut functioning in the non-diseased gut *in vitro.*

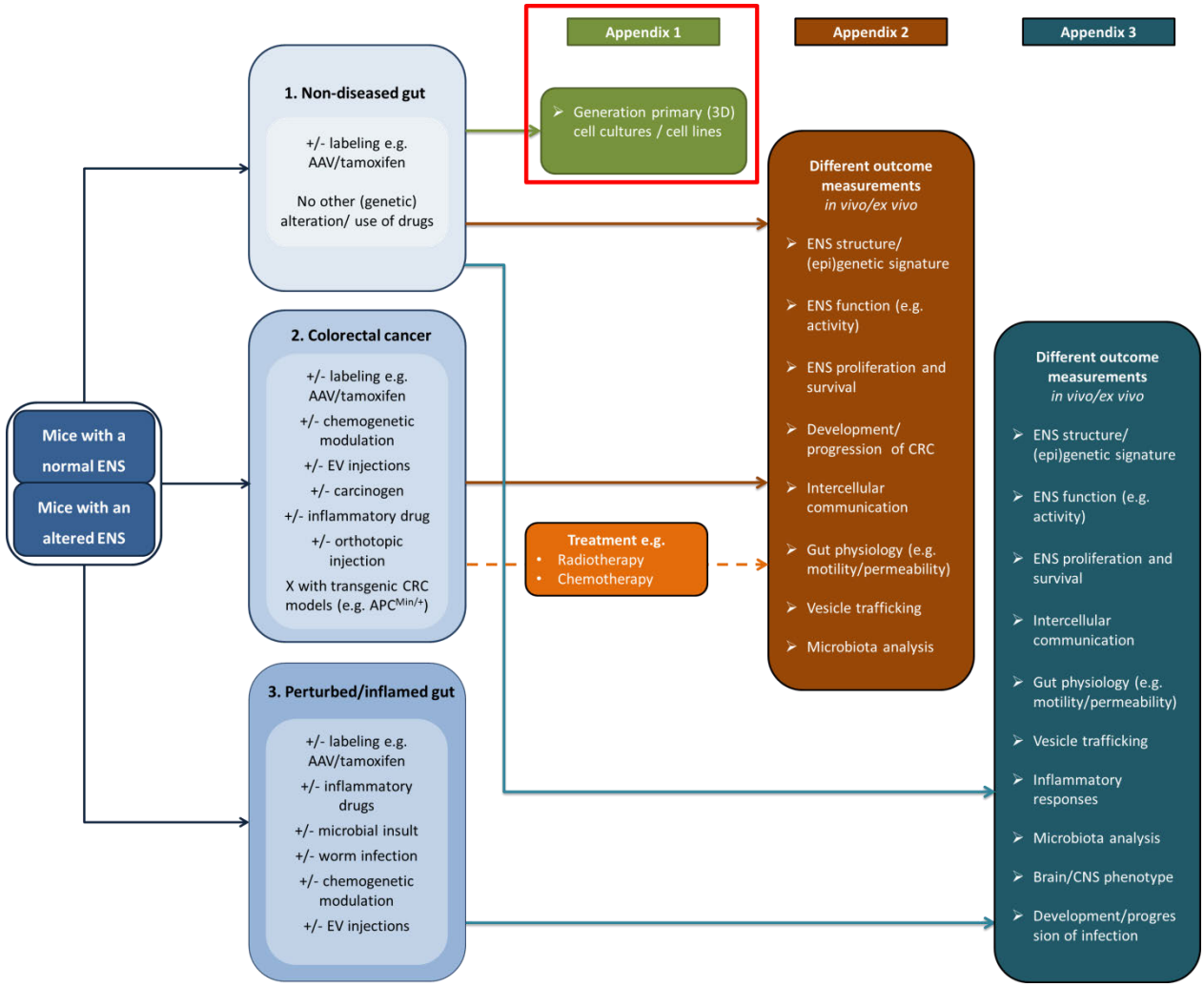

Figure 1. Outline depicting experimental models and outcome measures that will be assessed within each appendix. Red box delineates the purpose of this appendix.

Table 1: Mouse models and potential examples of mouse lines that can be used*:

Mouse model	Potential examples	Procedure
Wild-type mice		Possible saline injections Killing
Mice with fluorescently labelled ENS cells	Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}/Wnt1.Cre:R26-GCaMP6f	Tamoxifen injections (for SOX10.CreER^{T2}) Killing
Mice with fluorescently labelled extracellular vesicles from ENS cells	**CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre	Tamoxifen injections or AAV injections Killing
Transgenic mice with altered ENS	NDRG4$^{fl/fl}$Wnt1.Cre (ENS specific Ndrg4 knockdown) NSE-Noggin (more enteric neurons) Hand2$^{fl/+}$:Wnt1.Cre (less enteric neurons)	Killing
Lines designed to specifically modulate ENS activity	Sox10.CreER^{T2}/Wnt1.Cre:R26-hM4Di-DREADD	Tamoxifen injections (for SOX10.CreER^{T2}) Killing

* The mouse lines depicted within this table are at present the best models to investigate our research question. However, we would like to point out that better models might appear in the future. Consequently, the lines given here represent potential examples, by which means we will be able to change to better models in case these will be designed and/or become available. However, the number of models will remain the same (justification for mouse lines under section B – genetics alterations).
**X/X = flox/flox, flox/+ or littermate wildtype controls +/+

NB - All genotypes that are bred but are not going to be used for the procedures described in Appendix 2 and 3 can be used in this appendix for culturing cells and tissues, to follow the principles of 3Rs and reduce the number of bred animals.

In the course of the experiments, we will need neonates (P1-P3) of these mouse lines for the generation of enteric neurospheres. This is further specified in the sections below.

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

To investigate the role of ENS cells (e.g. vesicle secretion/ENS activity/miRNA) in the gut and their interactions with epithelial/immune/cancer cells (co-cultures with available cell lines), offspring obtained from the different mouse lines described above will be sacrificed for the generation of primary, mature (3D) cell cultures. In case of culturing progenitor cells and neurospheres, neonate mouse offspring will be used.

The animals (after the neonate stage) may be subjected to administration of substances (e.g., tamoxifen in the Sox10.CreERT2 mice) and viral particles (e.g., xAAVs for specific outcome measures) to label or modulate ENS cells, organelles and cell events depending on the mouse line used (see table 1; applies to ~70% of mice and further explained in section B). For labelling ENS cells: 1/ when using inducible Cre recombinase (e.g. Sox10CreERT2), recombination will be induced by one or two injections of tamoxifen (intraperitoneal), 2/ one intravenous injection of xAAVs will be performed. For labelling cell cycle events (e.g. BrdU), no more than one intraperitoneal injection of substances will be performed.

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

The number of animals we will need is based on our experience regarding cell culture techniques, our newly optimized protocol and related to the number of experiments that will/can be performed per week and per year, as obtained with our previous PL (AVD5.1 lid2h).

Importantly, using our optimized protocols, we have created a consistent and reliable way to culture ENS cells, neurospheres and organoids. Based on these protocols and experience, we calculated the numbers of animals we will need to statistically assess the outcomes of this study. These calculations are further explained below (B. number of animals).

ENS (enteric neurons, EGCs, progenitor cells), (and/or) adjacent normal epithelium will be preferentially obtained from the unused littermates coming from the breeding of the transgenic mice needed in appendix 2 and 3.

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
1	Mouse	In-house breeding	Adult & Neonate mouse offspring	1322	Male & female	Wildtype, Mice with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells, Transgenic mice with altered ENS, specifically modulated ENS activity	Specified below

Provide justifications for these choices

Species	Mice are used due to their anatomical, physiological, and genetic similarity to humans as well as their small size, ease of maintenance, short life cycle, and abundant genetic resources but particularly due to the established *in vitro* cell culture protocols

Origin	CD63 floxed from a licensed non-commercial breeder in USA, Sox10.CreERT2:R26tdTomato from a licensed non-commercial breeder within EU, Sox10.CreERT2/Wnt1.Cre:R26-GCaMP6f and Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD from licensed non-commercial breeders within Europe due to contributions/collaborations from other groups/institutes that share expertise, interests, genetically manipulated animal models. The remaining models are in house or will be obtained from a registered breeding facility.
Life stages	Adult – due to well-established *in vitro* protocols (Boesmans, Lasrado et al. 2015) (5.1 lid2e, 5.1 lid2h). Enteric neurospheres will be obtained from P1-P3 mouse of wildtype and transgenic mice (e.g. Wildtype, Wnt1.Cre:R26^{tdTomato}).
Number	In order to generate homogeneous and mixed ENS cultures (e.g., EGCs, neurons and progenitors), (and/or) adjacent normal epithelium, and intestinal organoids we need to isolate murine gastrointestinal tract. These cells will be used to investigate the role of ENS (ENS activity/ENS characteristics/vesicle secretion) in the gut, and their interplay with epithelial/tumor/immune cells (co-cultures with available cell lines). <u>ENS cultures:</u> Primary ENS cells will only grow for a maximum of 10 days and they do not divide, so we will need to isolate these cells every week. We prefer to isolate these cells from Sox10.CreERT2:R26tdTomato animals, as for most, but not necessarily all, experiments, we need fluorescently labelled ENS cells. In case we cannot generate decent/enough cultures using this line, we will (also) use wildtype animals. Given that with our optimised protocols we need the intestine of 2 mice to generate enough cells for one culturing procedure, will need 2 mice every week for 5 years, leading to a total of maximally **500** animals. Furthermore, other mouse lines with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells and transgenic mice with altered ENS or specifically modulated ENS will be used to describe specific cellular and molecular mechanisms for cell-to-cell communication. Therefore, we will need approximately 50 mice per genotype. We will use a maximum of 11 models/genotypes (genetically altered models described below and wildtype littermates for ENS altered models), so in total we will need **550** animals for this part. <u>Neurospheres:</u> Isolated neurospheres can be cultured for approximately 100 passages, so only a limited number of P1-P3 mouse neonates will be needed, as P1-P3 intestines contain a large number of proliferative ENS cells. However, we take into account that the isolation of neurospheres is a very new field, so protocols still have to be optimized using pilot experiments. From previous experiments performed by our collaborators, we know that a minimum of 8-10 P1-P3 intestines is required for each neurosphere isolation, so we need approximately 10x10 P1-P3 = 100 per genotype. For this aim we will use the Wnt1.Cre:R26^{tdTomato} line and (possibly) wildtype mice (see table 1), so **200** animals in total for this part. <u>Organoids:</u> Using previously adapted protocols from an experienced lab and Stem Cell technologies, we are able to generate abundant OGs from one intestinal section. OGs can be passaged/cultured for about 100 passages (12 mice per genotype for 5 years). For this aim we will be using NDRG4fl/fl:Wnt1.Cre, NSE-Noggin and Hand2fl/+:Wnt1.Cre mice with corresponding wildtypes (see table 1), so a total of **72** (12x6) animals. **Total: Max** 1322 mice
Gender	Both male and female mice will be used to minimise any gender-related variations in the experiment.

Genetic alterations	<table><tr><th>Mouse model</th><th>Potential examples</th><th>Readout</th><th>Number</th></tr><tr><td>Mice with fluorescently labelled ENS cells</td><td>Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}/Wnt1.Cre:R26-GCaMP6f</td><td>Creation of primary cell lines, neurospheres</td><td>500 + 100 200</td></tr><tr><td>Mice with fluorescently labelled extracellular vesicles from ENS cells</td><td>CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre</td><td>Creation of primary cell lines</td><td>50</td></tr><tr><td>Transgenic mice with altered ENS + corresponding WT</td><td>NDRG4$^{fl/fl}$:Wnt1.Cre, NSE-Noggin Hand2$^{fl/+}$:Wnt1.Cre</td><td>Creation of primary cell lines, organoids</td><td>300 72</td></tr><tr><td>Lines designed to specifically modulate ENS activity + corresponding WT</td><td>Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD</td><td>Creation of primary cell lines</td><td>100</td></tr></table> In the table above, the overall mouse models are described with specific examples of mouse lines that we want to use for this. However, as explained above, these lines are for now termed examples because we want to have the option of choosing a better model if those will be designed in the future. Each experimental model has its own purpose as different cell types/molecules will be labelled or present with different ENS-specific alterations. The specific reason and goal for the example lines that will be used to describe specific cellular and molecular mechanisms for cell behaviour and cell-to-cell communication are: • Sox10.CreERT2:R26tdTomato and Wnt1.Cre:R26tdTomato to label EGCs and/or enteric neurons • Sox10.CreERT2/Wnt1.Cre:R26-GCaMP6f for labelling and to investigate activity of EGCs and/or neurons • CD63f/+:Sox10.CreERT2:R26tdTomato or CD63f/f:Sox10.CreERT2:R26tdTomato to label, deplete and trace extracellular vesicles derived from EGCs • NDRG4fl/fl;Wnt1.Cre as enteric neuronal-specific knockdown of NDRG4 • NSE-Noggin and Hand2fl/+:Wnt1.Cre to study the effect of more respectively less enteric neurons • Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD to modulate ENS activity
Strain	(mixed) C57BL/6J (e.g. NDRG4fl/fl;Wnt1.Cre, Hand2fl/+:Wnt1.Cre, Sox10.CreERT2, R26-hM4Di-DREADD, R26-GCaMP6f), (mixed) 129S4SvJaeJ (e.g. Hand2fl/+:Wnt1.Cre), (mixed) FvB (e.g. NSE-Noggin). The strain of the wildtype mice will be matched to the corresponding transgenic mise used in the procedure.

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

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.

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

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

Genetically altered models:
Whereas Yoshimura et al described that adverse effects can occur in transgenic rats overexpressing human CD63-GFP (Yoshimura et al., Sci Rep., 2016), Men and colleagues have not reported CNS aberrations in mouse after inducible CD63 manipulation (Men et al., Nature Communications, 2019). This is in line with the observation of Yoshimura et al that the adverse effects of CD63-GFP overexpression can be circumvented by promoter-directed expression of the transgene in specific cell types of interest – similar as the procedure that will be applied here. Consequently, we do not expect any adverse effect when manipulating CD63 in the ENS. No adverse phenotype has been reported for the *Sox10.CreER*^{T2} mice (Laranjeira et al, JCI, 2011). Even though previous studies have described that the Hand2fl/+ x Wnt1.Cre mice might experience slowed gastrointestinal motility and constipation (Dáutreaux et al, Gastroenterology 2011), we now already have the mice for 2 years in our animal facility and we (researchers and CPV personnel) haven't observed this discomfort. Therefore, we don't expect to observe this harmful phenotype in this model.
For all the other lines described above, there are no data available describing adverse effects. However, based on the fact that the other models do not depict adverse effects, and that most of them merely represent reporter lines, we do not expect the appearance of deleterious phenotypes. Nevertheless, all animals will be closely monitored, and further actions (see pain relieve or humane endpoints) will be taken in case of change. To reduce stress for mothers of which we need neonates, we will set up the breeding in such way that we only have to use part of the litter for neonate experiments. The other pups will stay with the mother for normal length of time and will be used for breeding or experiments in the adult stage.

Administration of substances:
Based on experience and literature, we do not expect significant adverse effects from administration of transgene altering agents (e.g. tamoxifen) at any developmental stage. No more than transient discomfort caused by injection (max 2 times) at time of administration and no lasting harm are expected.
In case of effects in individual animals of particular scientific interest, the designated veterinarian will be requested for advice.

Explain why these effects may emerge.

NA

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

Animals will be monitored daily by experienced people (Animal facility staff (caretakers/ biotechnicians) or responsible investigators). The mice will be adequately housed and working procedures will be adjusted. Experimental procedures will be done aseptically.

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.

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).

Mild (100%):
- Administration of substances and labelling agents can cause short lasting mild pain and discomfort (transient)
- Mice will be killed after the protocol, where after we collect tissues to isolate/prepare primary (3D)cell cultures.

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	If possible, we will perform the experiments using established *in vitro* cell lines. Only when these techniques are not optimal to investigate our research questions, or if they do not work, will we use the primary cell cultures to investigate our hypothesis. For example, no ENS cell lines are available to study our research questions, therefore primary cell cultures need to be used. We aim to validate our data using cell cultures derived from human intestinal tissue specimens. However, compared to the high murine cell yield and tissue availability, a limited number of human tissue samples is available and the cell yield is also narrow, thereby limiting the possibility to adequately and preferentially use human samples. Furthermore, human samples cannot be easily manipulated to have the same potential as our mouse lines.
Reduction	We will limit the number of animals by isolating different cell types from the intestinal tract of the same mouse and by using animals that come from breedings carried out for the procedures in appendix 2/3, but cannot be used there.
Refinement	We do not expect to culture cells from any animals that display harmful phenotypes. Our experimental *in vitro* approach will allow us to gather very specific insights prior to doing any *in vivo* procedures.

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.

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.

N/A

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.

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.

N/A

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.

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.

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

It is necessary to kill the animals to extract cells to start primary (3D) cell cultures.

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.

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

☒ No > Describe the method of killing.

CO_2 inhalation and cervical dislocation

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

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

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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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

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.

Using the animal lines (table 1), models and procedures specified in this appendix, we aim to address RQ2 of our PL and investigate/ unravel the interaction of ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal cells (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (e.g. microbiota, CNS) in health and colorectal cancer (CRC) *in vivo*. Hereby, we will explore the interaction between the ENS and CRC and the potential underlying mechanisms.
In addition, we will isolate tumors and adjacent normal epithelium, to generate intestinal (tumor) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact intra- and extracellular mechanism by which the ENS affects the epithelial and tumor cells. In case of success in acquisition and analysis of data using these models (e.g. differences in cancer induction (tumor number/size/growth) between genetic models) (go/no-go), we will also study the potential impact of an impaired ENS (genetically/chemically-induced alteration) on current treatments of CRC (e.g. radio- and chemotherapy). We will use wild-type mice and mice with a labelled or altered ENS (see table below) containing a non-diseased gut (control) and/or a diseased gut (colorectal cancer) (**Figure 1**).

Mouse lines:

Table 1: Mouse models and potential examples of mouse lines that can be used*:

Mouse model	Potential examples
Wild-type mice	

Mice with fluorescently labelled ENS cells	Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}/Wnt1.Cre:R26-GCaMP6f
Mice with fluorescently labelled extracellular vesicles from ENS cells	CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre
Transgenic mice with altered ENS	NDRG4$^{fl/fl}$Wnt1.Cre, (enteric neural specific Ndrg4 knockdown) NSE-Noggin (more enteric neurons) Hand2$^{fl/+}$:Wnt1.Cre (less enteric neurons)
Lines designed to specifically modulate ENS activity	Sox10.CreER^{T2}/Wnt1.Cre:R26-hM4Di-DREADD

* The mouse lines depicted within this table are at present the best models to investigate our research question. However, we would like to point out that better models might appear in the future. Consequently, the lines given here represent potential examples, by which means we will be able to change to better models in case these will be designed and/or become available. However, the number of models will remain the same.

Several primary outcomes **(see figure 1 – orange box)** will be analysed by using one, or a combination of the animal models described above (table 1)**:**

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)
- ENS function (e.g., ENS connectivity and network activity)
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)
- Development/progression of CRC (e.g., track tumour burden)
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)
- Microbiota analysis (e.g., composition and abundance, bacteria secretome)

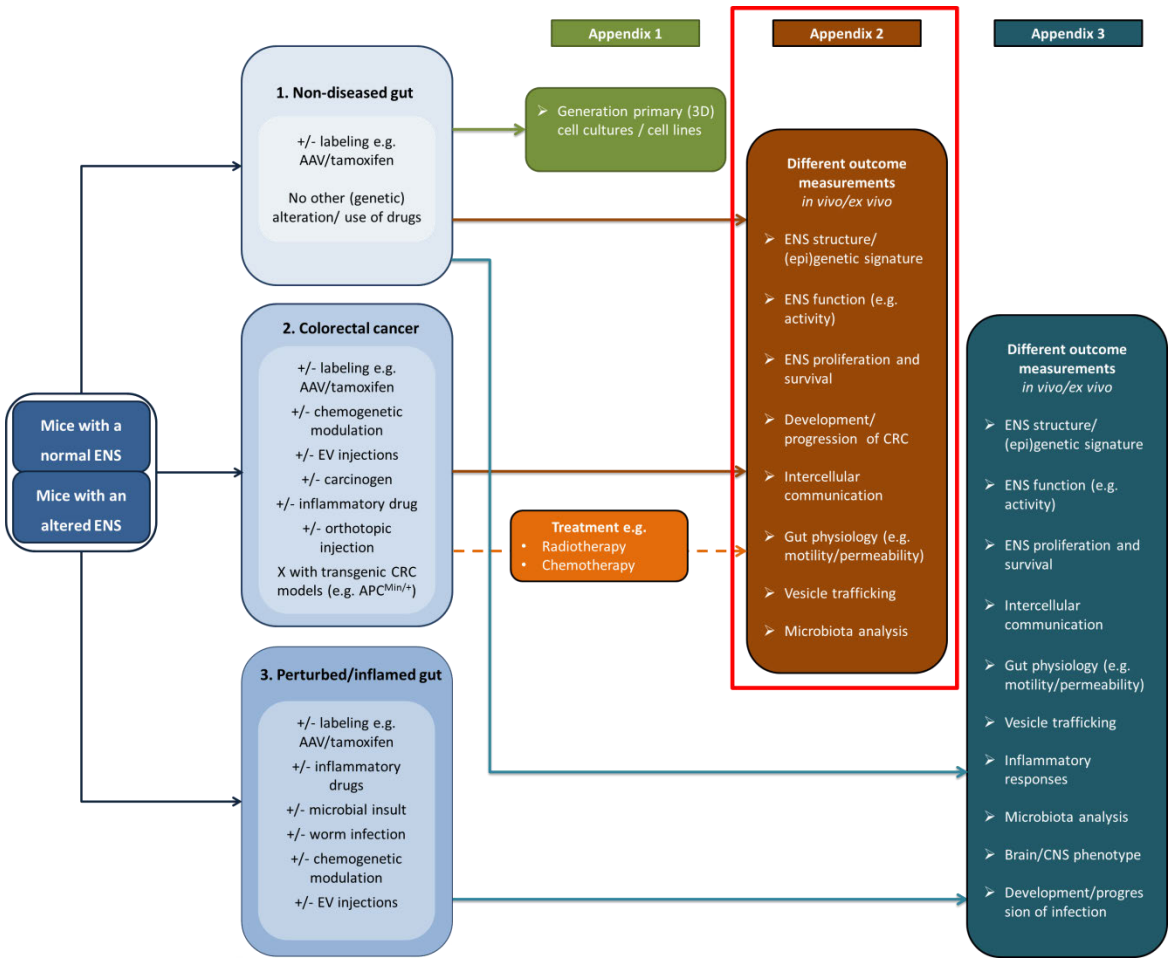

Figure 1. Outline depicting experimental models and outcome measures that will be assessed within each appendix. Red box delineates the purpose of this appendix.
With the experimental models and outcomes, we expect to further unravel mechanisms by which the ENS influences/responds to neighbouring environment in colorectal cancer by addressing the sub aims pointed below.

Aim 2: Investigate cellular and molecular mechanisms of interaction between ENS, intestinal (cancer) and other cells in a cancerous environment
Aim 2a: The role of EGCs in the development, progression, and treatment of CRC
Aim 2b: The role of enteric neurons in the development, progression, and treatment of CRC

This research will follow up on the research carried out under our previous project license 2017-026. Whereas animal lines, disease models and procedures partially overlap with PL2017-026, we have prevented as much overlap as possible. This is visualized in the table with all mouse numbers (word document – Total number of mice appendix 2) – where we omitted from the calculation of number of animals, the experiments/procedures that have already been performed. The data of these experiments demonstrate that the mouse lines and disease models are appropriate for studying the same outcome measures in other mouse lines and new outcome measures on both already in use and new mouse lines (see Figure 1 in PL on page 3 and figure 2 below). The only overlap in animals that is present in both licenses is the therapy part as there is not enough time on the old project license to complete these experiments. This is due to the delay in establishing the correct mouse models and the Covid-19 pandemic.

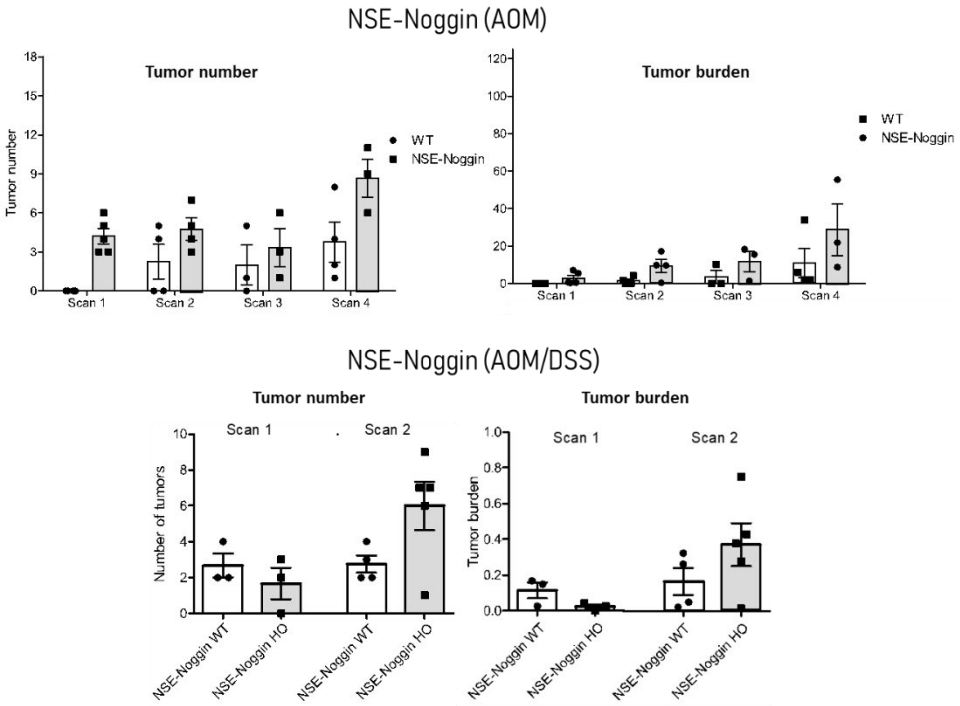

Figure 2: *CT scan results from pilot experiments of mice with normal number of neurons (WT) or more neurons (NSE-Noggin) in the ENS that underwent either AOM CRC induction protocol of AOM/DSS CSC induction protocol.* NSE-noggin (AOM): mice with more neurons seem to develop tumours faster and have a higher tumour burden at end stage than WT littermates. NSE-Noggin (AOM/DSS): onset of cancer seems to be similar, but tumour number and burden seem to be increased in mice with more neurons compared to WT.

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

To study how the ENS interacts with other cells, tissues and systems in health and CRC *in vivo* and *ex vivo*, we will take advantage of the well-established mouse lines described above (table 1). Animals can be subjected to CRC **(APC$^{Min/+}$, AOM, AOM/DSS, or orthotopic CRC model),** (epi)genetic editing (**AAVx-carrying genetic modifiers)**, and/or chemogenetic modulation **(DREADDs)** as described in the details below and in their respective programs of work. The same lines can be further examined after treatment for cancer **(radio- and chemotherapies)**.

Animal procedures for specific mouse models mentioned in table 1:

1. **Genetic editing of the ENS using viral vector transduction (AAVx.transgene) –** AAVs will be used to deliver genetic material to the ENS of mice to induce genetic alterations (e.g., deliver fluorescent reporters and/or silence, knockdown or overexpress genes) by e.g. tail vein injection, to analyse the response of the intestinal and extra-intestinal tissues.

2. **Chemogenetic modulation using designer receptors exclusively activated by designer drugs (DREADDs) -** AAVx vectors will be injected (e.g. tail vein injection) to target designer receptors exclusively activated by designer drugs (DREADD) variants to modulate ENS activity (e.g., activate or inhibit EGCs/enteric neurons). ENS structure, function and intercellular communication as well as gut physiology can be subsequently analysed**.** Similarly, DREADD receptors will be targeted to ENS cells using Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD mice. Receptor activation will be achieved by providing clozapine-N-oxide (CNO) to the animals (via drinking water or ip injections).

Animal procedures for non-diseased or control vs diseased mice using the mouse models described in table 1:

1. **Control group** (e.g., untreated/sham mice and/or homozygous wildtype control with no expected deleterious phenotype)
    - For the mice with fluorescently labelled ENS cells and fluorescently labelled extracellular vesicles from ENS cells, this will be untreated/sham mice (so no cancer induction), but containing the labelled ENS cells or EVs.
    - For the transgenic mice with altered ENS and the lines designed to specifically modulate ENS activity, this will be (homozygous) wildtype controls that underwent the same procedures/colorectal cancer induction protocols as the transgenic mice.

2. **Colorectal Cancer (CRC) –** The following models will be used to trigger the onset of CRC.
    - *Genetic model:* $APC^{Min/+}$ mice spontaneously develop tumours predominantly in the small intestine within approximately 6 months. Based on previous observations with our NDRG4 mice, we expect the development of about 30 tumours in all models, resulting in mild discomfort.
    - *Chemical carcinogen model*: Young adult mice (1-3 months) will be introduced to a well-established AOM carcinogen protocol (1 ip injection per week for 6 consecutive weeks). Tissues will be collected and analysed four months after the last AOM injection. Based on our previous observations by using this protocol, we expect the development of about 2-10 small colonic polyps in the models, causing mild discomfort.
    - *Chemical inflammatory/carcinogenic model:* Mice will be exposed to a combination of the inflammatory drug DSS (in drinking water) and the carcinogen AOM (ip injection), giving rise to colitis-associated cancer. Based on previous experience, we expect moderate discomfort and the appearance of about 10-40 colonic polyps, in the combined AOM/DSS model, but the development/growing of these polyps is faster than the AOM only model.
    - *Orthotopic CRC model:* Tumour cells (e.g. MC38 or CT26 cells) will be injected into the colon using a colonoscopy device when mice are under anaesthesia. A tumour will arise in every injection site (maximally three per mouse). Tumour metastasis and response to therapy (chemotherapy/radiotherapy) can be assessed in this model by CT imaging and evaluation after killing. Moderate to severe discomfort is expected.
      ***To evaluate the contribution of the ENS to the onset and progression of the disease, mice may also be killed prior to the appearance of polyps. However, CT scans can also be used for this purpose.**
      *** All CRC models used in this study do not have metastatic potential, so the mice will only develop tumours within the colon or small intestine, depending on the model used and the injection site.**

All four models have their specific characteristics and (dis)advantages. However, the chemical AOM (CRC model) and AOM/DSS (colitis-associated CRC model) model are the preferred models, because of the faster and more efficient CRC development. Nevertheless, we will apply the genetic and orthotopic models to answer specific questions like labelled cell tracking and specific insights in human-specific CRC pathogenesis. In addition, we will treat mice with CRC with either radiotherapy, chemotherapy, or a combination thereof which can give us more insight into the mechanisms of treatment effects and the patients that would benefit from these treatments. Controls will receive sham therapy.

- Radiotherapy: Irradiation (non-lethal dose; therapeutic efficacy) onto the tumor tissue (via CT imaging): frequency: Max to be determined (range 5-10); Duration: max 30 min. Further details: at different time points after CRC induction, mice will be anesthetized for micro-CT imaging. The target intestinal tumors will be delineated, where after a beam will be placed as such to cover the target and to ensure optimal sparing of organs at risk. Around the tumors, a 1-mm margin will be included to ensure also irradiation of microscopic disease spread. These procedures will take about 15 minutes in anesthetized animals. Upon obtaining the

optimal treatment plan, the target will be irradiated. After the procedures, animals are allowed to recover. Moderate (to severe) discomfort is expected from these procedures.

- Chemotherapy: Administration of for example 5-FU + oxaliplatin (at present the most used treatment for CRC) or saline: i.p. injection, dose per injection to be determined, max 10 injections. Moderate to severe discomfort is expected from these procedures.
- Combination treatment: treatment with radio- and chemotherapy, dosage and duration to be determined after the experiments with single application of radio- and chemotherapy are performed. Moderate to severe discomfort is expected from these procedures.
  Of Note: Pilot experiments for establishing appropriate doses of chemo- and radio-therapy are included and will be performed using our previous PL (AVD5.1 lid2h) to minimize the discomfort. By carrying out the pilot experiments, we will try to make sure that we are able to find the most efficient dose and minimize the discomfort to moderate. However, given that these pilot experiments have not yet been performed, we have now taken into account the worst-case scenario, wherein animals may experience severe discomfort.

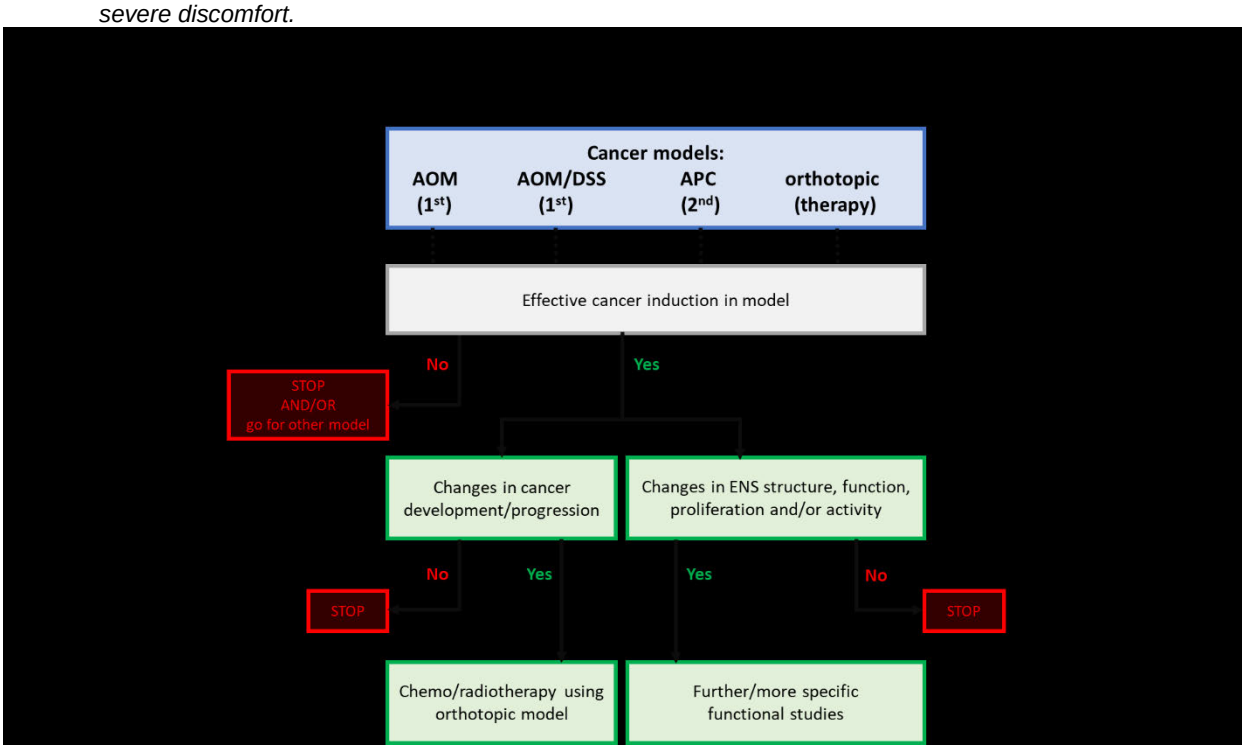

Figure 3. *Flowchart depicting the strategy and decision moments of the models (described above)/outcome measures (described below) in appendix 2.*

OUTCOME MEASUREMENTS THAT WILL BE ANALYSED:

As mentioned above, we will perform *in vivo* and/or *ex vivo* (post-mortem) analysis as follows:

NB – Many of the outcome parameters can be analysed by using one group of animals (e.g., ENS structure, intercellular communication, gut physiology and microbiota analysis). Support on the group sizes and total number of animals can be found at section B. The subdivision of number of animals per procedure/model and the total number is depicted in detail within the following Word document: Total number of mice; table – appendix 2 – wherein we refer to the similar enumeration as used below in our explanation. As explained before, the mouse models in these tables are used as examples but can be substituted if better models become available.

- ***ENS structure/ (epi) genetic signature & ENS function & ENS proliferation and survival***

  Distinct imaging techniques will be used to study these parameters on ENS (and its counterpart, the CNS).

- We will examine the influence of CRC on the ENS (and CNS) composition, architecture, morphology and activity (**ex vivo)**:

a) ***Fate mapping experiments*** - we will use *Sox10.CreER^{T2}:R26td^{Tomato}*. Tamoxifen (i.p.) will be injected at/before the time of initiation of the CRC protocol to label Sox10 expressing EGCs with tdTomato reporter. **– 12 animals per group.**

b) ***Calcium imaging of ENS activity*** - We will examine the ENS activity *ex vivo* in different ways: i) [Ca2+]i-Fluo-4 imaging: Live recording of ENS activity will be performed using Fluo-4 Ca2+ -imaging in preparations of whole-mount mouse gut. ii) For the other Ca2+ imaging experiments we will use Sox10-CreERT2::R26-GCaMP6f mice (EGCs, tamoxifen injection in adult animals) and Wnt1.Cre:: R26-GCaMP6f mice (all enteric neurons and glia). **– 12 animals per group**

- We will examine the influence of CRC on the ENS regarding cell proliferation and survival

c) **For cell proliferation and survival:** Analysis of ENS proliferation at different stages during CRC development/progression. Animals will be given a thymidine analogue (e.g. BrdU, edU) to label cycling cells via i.p. injections and/or drinking water. *In vivo/ex vivo* labelling assays will be used to detect cell death, senescence, DNA damage. We will perform this experiment only if we observe any phenotype in organisation, composition and function of the ENS. **– 12 animals per group**

- We will investigate the molecular signature of ENS cells and/or extracellular components in gut homeostasis and CRC. We will use FACS to isolate ENS cells for qRT-PCR, bulk or single-cell RNA Sequencing

d) **Tissue isolation, digestion and FACS**
Intestinal preparations will be dissociated into single cell suspensions and subjected to FACS for isolation of ENS cells and/or other components and analysis by qRT-PCR, bulk and single cell RNA Sequencing. **- 9 animals per group**

- ***Development/progression of CRC***

We will closely follow the onset and progression of tumours using the following techniques:

a) High resolution endoscopic monitoring - to analyse the effect of the treatment on tumour burden at different time points *in vivo*. **No culling, so performed in the mice that will be used at c.**
b) CT scans: This procedure will take about 15 minutes and will be done under anesthesia to prevent repositioning the animal. Contrast will be enhanced to visualize tumors using a protocol established in our lab and consists of an i.p. injection and a rectal injection of contrast agents. **No culling, so performed in the mice that will be used at c.**
c) All other outcomes will be analysed ex vivo (tumour number, size, histology, RNA expression, protein levels, cell isolation, neuron/EGC tracing, blood sampling etc.) **12 animals per group** to study histology and **6 animals per group** to study RNA/protein/cells.
d) This outcome measure will also be examined for the animals subjected to chemo- and/or radiotherapy. However, we need pilot studies with a smaller number of mice to first optimize the procedure before starting the experiment.

- ***Intercellular communication – 6 and 9 animals per group***
We will investigate cell-to-cell communication between ENS cells and other intestinal and extra-intestinal systems (e.g. immune system, tumour cells, brain) by immunohistochemistry, qRT-PCR, FACS, western blotting, ELISA, etc. Mice will be euthanised and intestinal and extra-intestinal tissues will be collected for subsequent analysis. We will also isolate tumors and adjacent normal epithelium, to generate intestinal (tumor) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact mechanism by which the ENS affects the epithelial and tumor cells.
NB: The tissue collection will be performed separately only when it cannot be achieved together with other experiments, in order to minimise unnecessary culling and animal use.

- ***Gut physiology (intestinal motility & gut permeability)***

- To study intestinal motility ***in vivo*** we will implement different methods:

a) *Total intestinal transit time*: The protocol for the total gastrointestinal transit time will be performed as previously described[5]. Mice will be individually placed into cages devoid of bedding and fasted for an hour. Next, a dye will be administrated by oral gavage, and the animals returned to their individual cages. The total intestinal transit time will be calculated by the time from gavage until the extrusion of the first coloured pellet. A maximum period of observation will be 5 hours in all experiments and mice that fail to expel the red pellet within this time will be quantified as ending point at 5 hours. ***NB: No culling is needed, and mice can be used in other read-out parameters. The dye is cleared from the gastrointestinal tract 2 days after administration. Faeces will be collected and analysed for bacterial composition and stool quality.**

b) *Small intestinal transit:* As described for the total transit time, mice will be given dye via oral gavage. Mice will be euthanised, followed by removal of the small intestine. The total length of the small intestine and the distance that the dye has travelled will be measured to determine the small intestinal transit length. ***Faeces will be collected and analysed for bacterial composition and stool quality. Tissues will be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain).**

c) Colonic propulsion: Colonic propulsion will be evaluated by first lightly anesthetizing the animals with isoflurane. A small diameter glass bead will be inserted into the rectum, and the expulsion time of the glass bead will be recorded. The assay will be repeated twice every 100 min for a total of 3 bead insertions. ***NB: No culling is needed, and mice can be used in other read-outs.**

NB – experiments in a and c can be performed using the same group of animals – 12 animals per group.

- **In case of a phenotype in a, b and/or c -** To study the intestinal contractility and motility ***ex vivo*** using live video recording and spatiotemporal analyses of ENS-dependent motility. Experiments can be performed using tissue derived from animals used in b.

d) Segments from distinct parts of the gut of young adult mice will be removed as previously described. Segments will be carefully isolated, luminal contents emptied and placed loosely pinned onto an organ bath chamber continuously infused with Krebs solution and constantly supplied with 95% O2 and 5% CO2 at 37°C and the neurogenic intestinal motility recorded.

- To study gut permeability ***in vivo***:

e) *Measuring levels of plasma FITC:* - Intestinal permeability will be determined by measuring levels of plasma FITC after administration via oral gavage of FITC-conjugated dextran in PBS. Blood will be obtained after administration, and the concentration of fluorescein will be determined by spectrophotofluorometry. Mice will be euthanized during this experiment. ***Faeces can be collected and analysed for bacterial composition and stool quality. Tissues can be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain). – 12 animals per group**

- ***Vesicle trafficking analysis***

- Distinct imaging techniques will be used to study ENS vesicle trafficking **– 12 animals per group**:

e) FM1-43 imaging ***(in vitro)***: We will investigate how synaptic signalling and synaptic vesicle turnover (FM1-43) differ in ENS cultures derived from the different mouse models by using FM1-43 imaging and after distinct stimuli (chemical, electrical).

f) NTA analysis ***(in vitro*** and ***ex vivo)*** - To analyse pattern parameters of EVs: intensity fluctuations, surface geometry and shape of the particles as well as particle concentration to distinguish sub-populations of vesicles.

g) Manipulation of EVs by viral vector targeting in the ENS ***(in vivo)*** - AAVx.XFP carrying genetic modifiers to silence (shmiRNA) or overexpress molecules of interest, will be injected (i.v.) in our animal models to evaluate the role of EVs in the maintenance, organisation and function of the ENS and in CRC. No side effects are expected with AAV

injections (mild discomfort). ***NB – Most of these experiments described above can be performed using the same group of animals as they are likely to cause transient and mild pain and discomfort.**

- ***Microbiota analysis***

Metagenomic DNA extraction from faecal, and tissue samples and molecular profiling/16S rRNA sequencing will be carried out to explore differences in the microbial community structure between experimental groups. We will examine the differences in microbial richness (e.g., *Chao1*) and diversity (e.g., *Shannon*). ***NB: The tissue collection will be achieved together with other experiments, in order to minimise unnecessary culling and animal use, so no extra animals will be used for this outcome measure.**

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

For the quantitative experiments, design has been based on ARRIVE guidelines and sample sizes have been set using power analysis https://www.statstodo.com/SSizCorr_Pgm.php. to determine the number of mice needed for each experiment, generally considering a difference between groups of at least 20%, power of 80% and significance level of 5%. For qualitative experiments, we will use the minimum number of mice to provide an accurate description based on previous publications and on our own experience, also from our previous PV.

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
1	Mouse	In-house breeding	(Young) adults	2118	Male and female	Wildtype, Mice with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells, Transgenic mice with altered ENS, specifically modified ENS activity	

Provide justifications for these choices

Species	Mice due to their anatomical, physiological, and genetic similarity to humans as well as their small size, ease of maintenance, short life cycle, and abundant genetic resources.
Origin	Sox10.CreERT2:R26tdTomato from a licensed non-commercial breeder in UK (but currently in house), Sox10.CreERT2:R26-GCaMP6f and *Wnt1.Cre:R26-hM4Di-DREADD* from licensed non-commercial breeders within Europe, NDRG4$^{fl/fl}$;Wnt1.Cre, NSE-Noggn and Hand2$^{fl/+}$;Wnt1.Cre are all in house, CD63 floxed from a licensed non-commercial breeder in USA
Life stages	Young adults (1-10 months) is most apt for optimal results
Number	Based on the literature, on our own and other collaborators experiences, we estimate that we will need a maximum of 12 mice per group for most outcome measures. For FACS experiments, we will need max 9 mice per group and 6 mice for RNA experiments. In total, this will be maximally 2118 animals. For a more detailed overview of how this number is built up, we refer to the following Word document: Total number of mice appendix 2.
Gender	Both male and female mice will be used to minimise any gender-related variations in the experiment.
Genetic alterations	Mice with fluorescently labelled ENS (e.g. Sox10.CreERT2:R26tdTomato, Wnt1.Cre:R26tdTomato, Sox10.CreERT2:R26-GCaMP6f) will be used to study the interaction between the ENS and CRC. Specific ENS cells will be labelled and can thereby be easily followed in these models. Furthermore, activity and

	specific ENS cells can be studied. This will give us more insight into the effect of CRC on specific ENS cells and their activity and therefore we will get a better idea about possible interactions between these cell types. Mice with fluorescently labelled extracellular vesicles from ENS cells (e.g. $CD63^{X/X}$ crossed with ENS specific cre reporter lines e.g., $Sox10.CreER^{T2}$ or Wnt1.Cre) can be used to study the involvement of extracellular vesicles in the interaction between the ENS and CRC. Transgenic mice with altered ENS (e.g., NDRG4fl/fl;Wnt1.Cre - Enteric neuronal-specific knockdown of NDRG4, Hand2f/+;Wnt1.Cre mice - more enteric neurons, and NSE-noggin - less enteric neurons) will be used to study whether neuron density or knockdown of NDRG4 (biomarker for CRC and specifically expressed in enteric neurons) affect CRC development/progression and which mechanisms play a role in this effect. Lines designed to specifically modulate ENS activity (e.g. $Sox10.CreER^{T2}$/Wnt1.Cre:R26-hM4Di-DREADD) will be used to study the effect of activation or inhibition of a specific subtype of ENS cells to the development/progression of CRC. This will give us insight into the role of ENS cells in CRC. Altogether, this may lead to possible pathways and/or targets that could be used for treatment in the future.
Strain	(mixed) C57BL/6J (e.g. NDRG4fl/fl;Wnt1.Cre, Hand2fl/+:Wnt1.Cre, Sox10.CreERT2, R26-hM4Di-DREADD, R26-GCaMP6f), (mixed) 129S4SvJaeJ (e.g. Hand2fl/+:Wnt1.Cre), (mixed) FvB (e.g. NSE-Noggin). The strain of the wildtype mice will be matched to the corresponding transgenic mice used in the procedure.

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

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.

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

During the development of CRC or during CRC treatment animals might experience pain (see below). All possibilities to reduce pain, fear or suffering will be used. These include use of appropriate analgesia and anaesthesia that do not affect gut motility, lead to gut abnormalities or affect the intestinal inflammatory response. Follow-up of the animals will be done regularly, at least daily, to ensure rapid notifications of signs of discomfort. Obviously, after experimental procedures e.g. injections/gavage, the animals will be followed up more frequently.

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

• *In vivo* experiments can cause distress or discomfort to the animals. Therefore, all animals will be frequently monitored for any signs of pain and distress. Changes to normal and provoked behaviour, movement, physical signs such as posture, respiration, skin and coat changes, inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist. Any mice exhibiting any deviation from the normal health and behaviour will be further monitored and treated accordingly.
• Genetically altered mice used in this appendix are not expected to show deleterious phenotypes.
• CRC mouse lines ($APC^{Min/+}$) and models (AOM and AOM/DSS) can develop side effects, such as pain, weight loss, diarrhoea/constipation and anal bleedings (no ulcers with perforation are expected).
• Radio- and Chemotherapies can induce necrosis, fibrosis and inflammation in irradiated tissues, and skin conditions may appear leading to loss of coat pigmentation depending on drug concentration. Furthermore, chemotherapy can induce side effects such as illness/sickness/nausea and reduction of appetite.

Explain why these effects may emerge.

• Most of the side effects mentioned above are unlikely in our non-diseased mice models (unless provoked), but can occur due to inflammation and/or progression of disease, development of polyps and alteration in intestinal microbiota.
• Radio- and chemotherapy can cause toxicity in normal/healthy tissues due to production of reactive oxygen/nitrogen species (ROS). This may result in immediate cell death, inflammation, tissue fibrosis and DNA damage in neighbouring tissues.

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

Animals will be frequently monitored by experienced staff and researchers. Our mouse colonies will be housed and maintained according to the basic guidelines for animal welfare. Working protocols will be adequately adjusted to benefit not only the research but also animal welfare. Experimental procedures/routes of administration, techniques that cause the least pain, suffering, distress and the shortest lasting harm will be preferred. Experimental procedures will be performed by using aseptic/sterile techniques, and drugs such as analgesics, anaesthetics and antibiotics will be administered whenever necessary. Examples of measures that we will use to prevent or minimise harm are minimizing radiotherapy and chemotherapy dosages and times to what is minimally required, extra hydration in case of diarrhea and boosting gels in case of high weight loss. Measures will be taken accordingly in the case any unexpected adverse effects might appear and animals will be immediately euthanized and experiments stopped in the case of unexpected severe signs of pain and discomfort.

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.

- All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist.
- Any animal will be immediately euthanised if it exhibits signs of suffering that is not transient or treatable, and compromises its normal behaviour.
- Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe discomfort and >20% for severe discomfort) that persist for 24 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision.
- Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled.
- Chemical CRC models: animals undergo euthanasia about 100 days after the beginning of the procedure. Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 2), “Wasting Syndrome” (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), or opportunist infections, the animals will be immediately euthanised.
- Radio- and Chemotherapies: adverse effects such as inflammation and skin conditions such as loss of coat pigmentation may appear. Animals can undergo moderate to severe adverse effects and will be culled if signs of severe discomfort persist such as facial expression score of 3, “Wasting Syndrome” (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), coat/skin condition 3 for at least 3 consecutive days, or opportunist infections.

Indicate the likely incidence.

For the CRC models, and in the wildtype x CRC mice that underwent chemo or radiotherapy (±10-15% incidence). The animals will be well monitored by experienced people, so that we are able to rapidly detect unforeseen adverse effects in early stages, whereby we aim to avoid reaching the humane endpoints.

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).

Appendix 2	Non-diseased gut (I)	APC$^{Min/+}$ mouse model (II)	AOM model (III)	AOM/DSS model (IV)
Discomfort model itself → Discomfort procedures per outcome measure ↓	**No discomfort**	**Mild**	**Mild**	**Moderate**
ENS structure /function /proliferation - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Gut physiology - Administration of substances: **mild** - Transit/motility assays: **mild** - Killing: **mild**	Mild	Moderate	Moderate	Severe
Vesicle trafficking - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Microbiota analysis - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Intercellular communication - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Development/progression of CRC - Administration of substances: **mild** - CT scans: **moderate** - Killing: **mild**	Moderate	Moderate	Moderate	Severe

Appendix 2: Treatment	AOM model (III)	AOM/DSS model (IV)	Orthotopic model (V)
Discomfort model + treatment (chemo/radiotherapy)→ Discomfort procedures per outcome measure ↓	**Moderate**	**Moderate**	**Moderate**
Development/progression of CRC - Administration of substances: **mild** - CT scans: **moderate** - Killing: **mild**	Moderate	Severe	Severe

Figure 4: *Cumulative discomfort for disease models or treatment models (columns) combined with outcome measurements and corresponding procedures (rows).* The different mouse lines itself (described in Table 1) are not expected to have any harmful phenotype or display signs of discomfort.

Approximate percentages expected cumulative discomfort based on animal numbers per outcome measure and disease model:
Mild: 50%
Moderate: 32%
Severe: 18%

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	To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models to study CRC. In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied *in vivo*. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established *in vitro* culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis *in vivo.* We are also taking advantage of recently established collaborations (e.g. in the gastroenterology clinic (5.1 lid2h)) to obtain human intestinal specimens and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study cancer development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Reduction	Before embarking on procedures in animal research, we will collect evidence form *in vitro/in silico* procedures if possible to determine whether a candidate gene or molecule also provide insights on *in vivo* pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This can also be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals (mostly included in the previous PL (AVD5.1 lid2h), so not included in the current strategy). This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.
Refinement	Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no phenotype and/or useful results, experiments that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and/or well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached.

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.

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.

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.

N/A

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.

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.

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

Ex vivo (post-mortem) and *in vitro* experiments will be performed in tissue and cells collected from our experimental groups to further analyse the biology of the nervous system.

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.

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

☒ No > Describe the method of killing.

CO_2 inhalation and cervical dislocation

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

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

Appendix 2							
Non-diseased gut (I)	***Sox10CreERT2/ Wnt1Cre-TdTomato (Ctrl AOM) (i)***	***Sox10CreERT2/Wnt1Cre-TdTomato (Ctrl AOM/DSS) (ii)***	***NDRG4$^{fl/fl}$ – Wnt1^{Cre2} (iii)***	***NDRG4$^{fl/fl}$ (iv)***	***APC$^{+/+}$ Sox10CreERT2-TdTomato (v)***		
ENS structure /function /proliferation 1. Number/morphology (a) 2. Activity (b) 3. Proliferation/Survival (c) 4. FACS (d)	#/measure →12 →9	#/measure →12 →12 →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →12 →12 →9		**153**
Gut physiology 5. whole-gut + colonic (a, c) 6. small intestinal transit + ex vivo motility (b, d) 7. permeability (e)	#/measure →12 →12 →12	#/measure →12 →12 →12	#/measure	#/measure	#/measure →12 →12 →12		**108**
Vesicle trafficking 8. Imaging (a, b, c, d)	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12		**60**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis		**0**
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →9	#/measure →6 →9	#/measure →9	#/measure →9	#/measure →6 →9		**57**
Development/progression of CRC 11. Histology (c) 12. RNA/protein/cell isolation (c)	#/measure	#/measure →12 →6	#/measure	#/measure	#/measure →12 →6		**36**
							414

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h .

Appendix 2								
***$APC^{Min/+}$* mouse model (II)**	***Sox10CreERT2 -TdTomato (v)***	***$NDRG4^{fl/fl}$ – $Wnt1^{Cre2}$ (iii)***	***$NDRG4^{fl/fl}$ (iv)***	***$Hand2^{fl/+}$ $Wnt1^{Cre2}$ (vi)***	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***	
ENS structure /function /proliferation 1. Number/morphology (a) 2. Activity (b) 3. Proliferation/Survival (c) 4. FACS (d)	#/measure →12 →12 →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	**171**
Gut physiology 5. whole-gut + colonic (a, c) 6. small intestinal transit + ex vivo motility (b, d) 7. permeability (e)	#/measure →12 →12 →12	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	**36**
Vesicle trafficking 8. Imaging (a, b, c, d)	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	**84**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	0
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →6 →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	**69**
Development/progression of CRC 11. Histology (c) 12. RNA/protein/cell isolation (c)	#/measure →12 →6	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	**18**
								378

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h .

Appendix 2									
***AOM* model (III)**	***Sox10CreERT2 /Wnt1Cre-TdTomato (i)***	***NDRG4$^{fl/fl}$ Wnt1^{Cre2} (iii)***	***NDRG4$^{fl/fli}$(v)***	***Hand2$^{fl/+}$ Wnt1^{Cre2} (vi)***	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***	***Sox10CreERT2:R26-hM4Di-DREADD or GCaMP6f (x)***	
ENS structure /function /proliferation 1. Number/morphology **(a)** 2. Activity **(b)** 3. Proliferation/Survival **(c)** 4. FACS **(d)**	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →12 →12 →9	**183**
Gut physiology 5. whole-gut + colonic **(a, c)** 6. small intestinal transit + ex vivo motility **(b, d)** 7. permeability **(e)**	#/measure →12 →12 →12	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →12 →12	**72**
Vesicle trafficking 8. Imaging **(a, b, c, d)**	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	**96**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	0
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →6 →9	**78**
Development/progression of CRC 11. Histology **(c)** 12. RNA/protein/cell isolation **(c)**	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →6	**18**
									447

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h .

Appendix 2									
***AOM/DSS* model (IV)**	***Sox10CreERT2/Wnt1Cre - TdTomato (ii)***	***NDRG4$^{fl/fl}$ Wnt1^{Cre2} (iii)***	***NDRG4$^{fl/fl}$ (iv)***	***Hand2$^{fl/+}$ Wnt1^{Cre2} (vi)***	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***	***Sox10CreERT2:R26-hM4Di-DREADD or GCaMP6f (x)***	
ENS structure /function /proliferation 1. Number/morphology (a) 2. Activity (b) 3. Proliferation/Survival (c) 4. FACS (d)	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →12 →12 →9	**183**
Gut physiology 5. whole-gut + colonic (a, c) 6. small intestinal transit + ex vivo motility (b, d) 7. permeability (e)	#/measure →12 →12 →12	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →12 →12	**72**
Vesicle trafficking 8. Imaging (a, b, c, d)	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	**96**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	0
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →6 →9	**78**
Development/progression of CRC 11. Histology (c) 12. RNA/protein/cell isolation (c)	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →6	**18**
									447

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h

***CRC treatment* models (V)**								
Chemotherapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+ AOM or***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***orthotopic***	→6	→6	→6	→6			**72**
11. Histology	***+ AOM/DSS***	→12	→12	→12	→12			
12. RNA/protein/cell isolation		→6	→6	→6	→6			**72**
Control chemotherapy – sham therapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+ AOM or***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***orthotopic***	→6	→6	→6	→6			**72**
11. Histology		→12	→12	→12	→12			
12. RNA/protein/cell isolation	***+ AOM/DSS***	→6	→6	→6	→6			**72**
								288

Radiotherapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***Orthotopic***	→6	→6	→6	→6			**72**
Control Radiotherapy – sham therapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***Orthotopic***	→6	→6	→6	→6			**72**
								144

TOTAL appendix 2 = 414 + 378 + 447 + 447 + 288 + 144 =	**2118**

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h .

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 3 Type of animal procedure: Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

In this appendix we aim to investigate and unravel the interaction between adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) and other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota, CNS) in health and/or upon local and systemic challenges (RQ3 of the PL).

Mouse lines:

Table 1: Mouse models and potential examples of mouse lines that can be used*:

Mouse model	Potential examples
Wild-type mice	
Mice with fluorescently labelled ENS cells	Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}:R26-GCaMP6f
Mice with fluorescently labelled extracellular vesicles from ENS cells	**CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre
Lines designed to specifically modulate ENS activity	Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD

* The mouse lines depicted within this table are at present the best models to investigate our research question. However, we would like to point out that better models might appear in the future. Consequently, the lines given here represent potential

examples, by which means we will be able to change to better models in case these will be designed and/or become available. However, the number of models will remain the same.

Several primary outcomes **(see figure 1 – dark blue box)** will be analysed by using one or a combination of the animal models described above**:**

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)
- ENS function (e.g., ENS connectivity and network activity)
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)
- Inflammatory processes (e.g., immune cell phenotype)
- Microbiota analysis (e.g., composition and abundance, bacteria secretome)
- Brain/CNS phenotype (e.g., organisation, non-invasive behavioural tests, imaging)
- Development/progression of infection (e.g., track parasitic infection and worm burden, numbers and eggs)

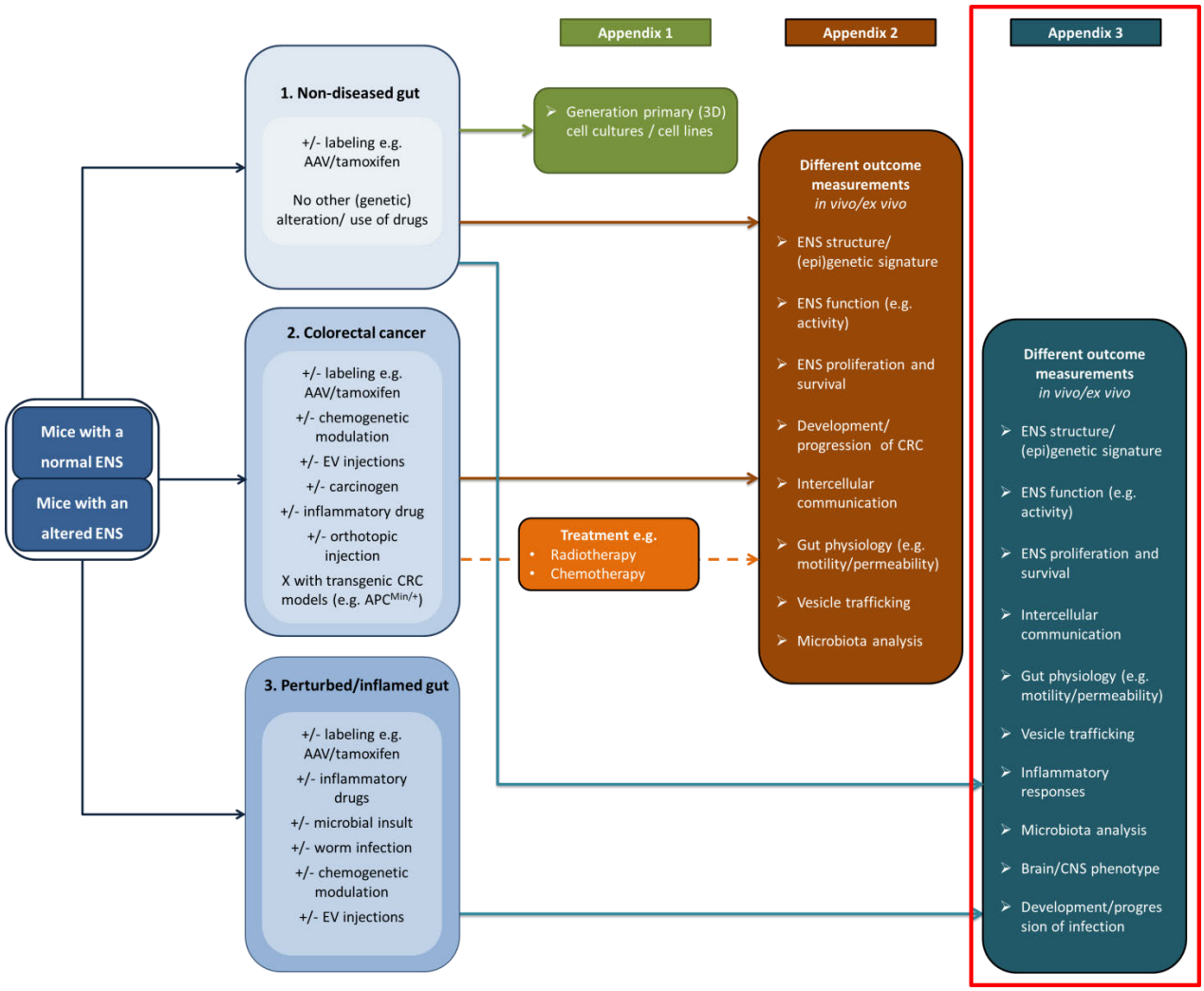

Figure 1. Outline depicting experimental models and outcome measures that will be assessed within each appendix. Red box delineates the purpose of this appendix.

With the presented experimental models and outcomes, we expect to further unravel mechanisms by which the ENS influences/responds to neighbouring environment by addressing the sub aims pointed below.

Aim 3: Investigate the mechanisms of cell-to-cell communication in homeostasis and diseases that affect the gastrointestinal tract (e.g., **worm infection**, (and/or) microbiota alteration, (and/or) inflammation).
Aim 3a: The role of ENS-derived EVs for cell-to-cell communication in health and disease.

Aim 3b: The role of ENS-derived miRNAs

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

To study how the ENS interacts with other cells/tissues and systems in health and disease *in vivo* and *ex vivo*, we will take advantage of the well-established mouse lines described above. Animals can be subjected to worm infection **Heligmosomoides polygyrus (*H. poly*),** (epi)genetic editing (**AAVx-carrying genetic modifiers)**, intestinal inflammation **(DSS/BAC),** microbiota alterations **(depletion/antibiotics),** chemogenetic modulation **(DREADDs)** as described in detail below and in their respective programs of work. The same lines can be further examined by rescue experiments to re-establish the intestinal microbiota composition **(reintroduction of microbes by faecal transplantation)**, rescue of specific phenotype by using advanced technology to deliver molecules **(AAVx.transgene).**

Animal procedures for specific mouse models mentioned in table 1:

- **Genetic editing of the ENS using viral vector transduction (AAVx.transgene) –** AAVs will be used to deliver genetic material to the ENS of mice to induce genetic alterations (e.g., deliver fluorescent reporters and/or silence, knockdown or overexpress genes) by e.g. tail vein injection, to analyse the response of the intestinal and extra-intestinal tissues. To our knowledge, literature doesn't describe a harmful phenotype induced by these injections, so there will be no more than mild discomfort.

- **Chemogenetic modulation using designer receptors exclusively activated by designer drugs (DREADDs) -** AAVx vectors will be injected (e.g. tail vein injection) to target designer receptors exclusively activated by designer drugs (DREADD) variants to modulate ENS activity (e.g., activate or inhibit EGCs/enteric neurons) (max. mild phenotype). ENS structure, function and intercellular communication as well as gut physiology can be subsequently analysed**.** Similarly, DREADD receptors will be targeted to ENS cells using Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD mice. Receptor activation will be achieved by providing clozapine-N-oxide (CNO) to the animals (via drinking water or i.p. injections). We will perform shRNA knockdown of ENS genes using AAVx vector technology to modulate ENS cells. To target critical stages of ENS network formation, these interventions can be performed during late prenatal, early postnatal stages of ENS development, or adult mice of either sex.

Animal procedures for non-diseased or control vs diseased mice using the mouse models described in table 1:

1) **Control group** (e.g., untreated/sham mice and/or homozygous wildtype control with no expected deleterious phenotype)
- For the mice with fluorescently labelled ENS cells, fluorescently labelled extracellular vesicles from ENS cells and the lines designed to specifically modulate ENS activity, this will be untreated/sham mice but containing the labelled/activity modulated ENS cells or EVs.

2) **Perturbed/Inflamed gut –** Various challenges will be used to affect gastrointestinal homeostasis and trigger an ENS response (see figure 1 for breakdown of go/no go decision moments):
- Worm Infection – *H. poly* are mouse pathogens. While wild-type mice are able to clear the worm without exhibiting significant clinical problems, mice carrying ENS-specific mutations may be more susceptible to pathology. Infected mice are expected to have mild discomfort. Mice will be infected with *H. poly* third stage (L3) larvae by oral gavage, which were obtained from faecal cultures of H. poly-infected mice from a collaborating lab (Johnston et al, J Vis Exp, 2015). The number of adult worms in the intestinal wall at day 7 of infection, the number of adult worms in the intestinal lumen and the number of macroscopically visible granulomas will be enumerated by manual counting (5.1 lid2e, 5.1 lid2h)
- Genetic editing: (Go/No-go) If we find specific targets of interest (e.g. microRNAs) that are dysregulated in the ENS in the worm infection model we will use advanced technology to deliver molecules (AAVx.transgene) – AAVs will be used to deliver genetic material to the ENS of mice to induce genetic alterations (e.g., silence, knockdown or overexpress genes) by e.g. tail vein injection, and thus, we can then analyse the response of the intestinal and extra-intestinal tissues.
- Intestinal Inflammation (systemic and focal): (Go/No-go) These models will be used if we find an immune related phenotype (e.g. Immune pathway activations, immune cell perturbances) in the worm infection model. In this case, we will use well-known chemicals that trigger systemic (dextran sodium sulfate – DSS; in drinking water) and focal

(benzalkonium chloride – BAC) intestinal inflammation. To induce acute or chronic inflammation, with different degrees of severity, animals will be subjected to DSS treatment (varying time frame and concentrations).
For BAC – BAC will be surgically applied onto the serosal surface of the intestine by laparotomy (distal ileum or proximal colon). Mice will be closely monitored, and tissues harvested after surgery. *To evaluate the contribution of the ENS to the onset and progression of the disease, mice will also be collected prior to the development of inflammation.

- Alteration in intestinal microbiota (Antibiotic treatment and microbiome reconstitution): (Go/No-go) These models will be used if we find alterations in the intestinal microbiota (e.g. disruptions in the communication between ENS and intestinal microbiota) in the worm infection model. In this case, young adult mice or pregnant females will be subjected to single or a cocktail of broad-spectrum antibiotics in their drinking water (chase) or oral gavage (pulse). The antibiotic cocktail consists of ampicillin, metronidazole, vancomycin and neomycin. The effect of antibiotics on intestinal microflora will be examined by quantitative analysis of aerobic and anaerobic bacteria and yeasts present in faecal pellets. No major side effects are expected from the administration of antibiotics in drinking water. However, animals will be closely monitored, and their body weight regularly recorded. *For flora reconstitution* - Prior to colonisation, faecal pellets used to reconstitute bacterial populations and the efficiency of bacteria inoculation will be confirmed by analysing the genomic DNA of total bacteria in faecal samples by qRT-PCR.

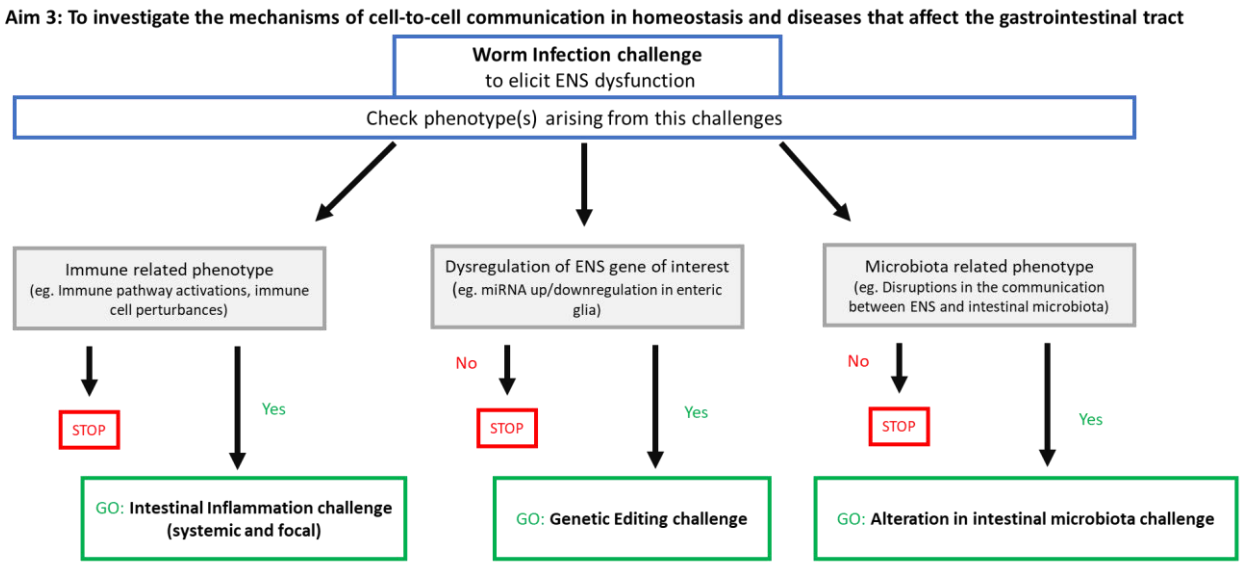

Figure 2. *Flowchart depicting crucial Go/No-go decision moments.*

NB – If dysregulation in the immune system, and/or the microbiome and/or ENS target genes arise from the worm infection challenge then animals can be subjected to more than one follow-up challenge (e.g. intestinal inflammation challenge and alteration in intestinal microbiota challenge)

OUTCOME MEASUREMENTS THAT WILL BE ANALYSED

As mentioned above, we will perform *in vivo* and/or *ex vivo* (post-mortem) analysis as follows:

NB – Many of the outcome parameters can be analysed by using the same group of animals (e.g., ENS structure, intercellular communication, gut physiology microbiota analysis and CNS phenotype). Support on the group sizes and total number of animals can be found at statistical methods and section B. Animals – Number (where we refer to the following Word document: Total number of mice; table – appendix 3). As explained before, the provided examples of mouse models in these tables are at present the preferable models, but we will substitute them if better models become available.

- ***ENS structure/ (epi) genetic signature & ENS function & ENS proliferation and survival***

Distinct imaging techniques will be used to study these parameters on ENS and its counterpart, the CNS:

We will examine the influence of challenges mentioned above (e.g., worm infection, genetic editing of the ENS, intestinal inflammation, alteration in intestinal microbiota) on ENS and CNS composition, architecture, morphology and activity (**ex vivo)**.

a) ***Fate mapping experiments*** - we will use *Sox10.CreER^{T2}:R26td^{Tomato}* in challenge models described above. Tamoxifen (i.p.) will be injected at the time of initiation of the challenging protocol to label Sox10 expressing EGCs with tdTomato reporter. **– 12 animals per group.**

b) ***Calcium imaging of ENS activity*** - We will examine the ENS activity *ex vivo* in different ways: i) [Ca2+]i-Fluo-4 imaging: Live recording of ENS activity will be performed using Fluo-4 Ca2+ -imaging in preparations of whole-mount mouse gut after various stimulations (chemical, electrical). ii) For the other Ca2+ imaging experiments we will use Sox10-CreERT2::R26-GCaMP6f mice (EGCs, tamoxifen injection in adult animals) and Wnt1.Cre:: R26-GCaMP6f mice (all enteric neurons and glia). **– 12 animals per group**

c) ***For cell proliferation and survival:*** Analysis of ENS proliferation at different stages of the challenges detailed above. Animals will be given a thymidine analogue (e.g. BrdU, edU) to label cycling cells via i.p. injections and/or drinking water. In vivo/ex vivo labelling assays will be used to detect cell death, senescence, DNA damage. We will perform this experiment only if we observe any phenotype in organisation, composition and function of the ENS. – **12 animals per group**

We will investigate the molecular signature of ENS cells and/or extracellular components in gut homeostasis and under intestinal challenge. We will use FACS to isolate ENS cells for qRT-PCR, bulk or single-cell RNA Sequencing

d) ***Tissue isolation, digestion and FACS***: Intestinal preparations will be dissociated into single cell suspensions and subjected to FACS for isolation of ENS cells and/or other components and analysis by qRT-PCR, bulk and single cell RNA Sequencing. **- 9 animals per group**

- ***Intercellular communication – 6 and 9 animals per group***

We will investigate cell-to-cell communication between ENS cells and other intestinal and extra-intestinal systems by immunohistochemistry, qRT-PCR, western blotting, ELISA, etc. Mice that underwent intestinal challenge (specified above) will be euthanised and intestinal and extra intestinal tissues will be collected for analysis. **NB: The tissue collection will be performed separately only when it cannot be achieved together with other experiments, in order to minimise unnecessary culling and animal use.**

- ***Gut physiology (intestinal motility & gut permeability)***

To study intestinal motility ***in vivo*** we will implement different methods:

a) ***Total intestinal transit time*****:** The protocol for the total gastrointestinal transit time will be performed as previously described[5]. Mice will be individually placed into cages devoid of bedding and fasted for an hour. Next, a dye will be administrated by oral gavage, and the animals returned to their individual cages. The total intestinal transit time will be calculated by the time from gavage until the extrusion of the first coloured pellet. A maximum period of observation will be 5 hours in all experiments and mice that fail to expel the coloured pellet within this time will be quantified as ending point at 5 hours. ***NB: No culling is needed, and mice can be used in other read-out parameters. The dye is cleared from the gastrointestinal tract 2 days after administration. Faeces will be collected and analysed for bacterial composition and stool quality.**

b) ***Small intestinal transit:*** As described for the total transit time, mice will be given dye via oral gavage. Mice will be euthanised, followed by removal of the small intestine. The total length of the small intestine and the distance that the dye has travelled will be measured to determine the small intestinal transit length. ***Faeces will be collected and analysed for bacterial composition and stool quality. Tissues will be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain).**

c) **Colonic propulsion**: Colonic propulsion will be evaluated by first lightly anesthetizing the animals with isoflurane. A small diameter glass bead will be inserted into the rectum, and the expulsion time of the glass bead will be recorded. The assay will be repeated twice every 100 min for a total of 3 bead insertions. ***NB: No culling is needed, and mice can be used in other read-outs.**

NB – experiments in a and c can be performed using the same group of animals – 12 animals per group.

In case of a phenotype in a, b and/or c - To study the intestinal contractility and motility ***ex vivo*** using live video recording and spatiotemporal analyses of ENS-dependent motility. Experiments can be performed using tissue derived from animals used in b.

d) Segments from distinct parts of the gut of young adult mice will be removed as previously described[6]. Segments will be carefully isolated, luminal contents emptied and placed loosely pinned onto an organ bath chamber continuously infused with Krebs solution and constantly supplied with 95% O2 and 5% CO2 at 37°C and the neurogenic intestinal motility recorded.

To study gut permeability ***in vivo***:

e) *Measuring levels of plasma FITC:* - Intestinal permeability will be determined by measuring levels of plasma FITC after administration via oral gavage of FITC-conjugated dextran in PBS. Blood will be obtained after administration, and the concentration of fluorescein will be determined by spectrophotofluorometry. Mice will be euthanized during this experiment. ***Faeces can be collected and analysed for bacterial composition and stool quality. Tissues can be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain). – 12 animals per group**

- ***Vesicle trafficking analysis***

Distinct imaging techniques will be used to study ENS vesicle trafficking **– 12 animals per group**:

a) FM1-43 imaging ***(in vitro)***: We will investigate how synaptic signalling and synaptic vesicle turnover (FM1-43) differ in ENS cultures derived from the different mouse models by using FM1-43 imaging and after distinct stimuli (chemical, electrical).
b) NTA analysis ***(in vitro*** and ***ex vivo)*** - To analyse pattern parameters of EVs: intensity fluctuations, surface geometry and shape of the particles as well as particle concentration to distinguish sub-populations of vesicles.
c) Manipulation of EVs by viral vector targeting in the ENS ***(in vivo)*** - AAVx.XFP carrying genetic modifiers to silence (shmiRNA) or overexpress molecules of interest (miRNAs), will be injected (i.v.) in our animal models to evaluate the role of EVs in the maintenance, organisation and function of the ENS. No side effects are expected with AAV injections (mild discomfort). ***NB – Most of these experiments described above can be performed using the same group of animals as they are likely to cause transient and mild pain and discomfort.**

- ***Inflammatory processes and immunophenotyping***

Gut inflammation will also be closely monitored ***in vivo*** (faecal analysis) and scored ***ex vivo*** by using **immunohistochemical** staining for inflammatory markers and immune cells, **H&E** and **flow cytometry (see below).** For the immunophenotyping, intestinal, lymphoid tissues and blood preparations will be dissociated, and single cell suspensions counted and pre-incubated with antibodies to label myeloid and lymphoid cells. Stained samples will be processed using a flow cytometer, and the obtained raw data analysed. Different cell populations will be identified by the general gating strategy and the total number of cells estimated by the cell count multiplied by the percentage of live cells- ***12 animals per group/timepoint*** ***NB These experiments can be performed using the same group of animals.**

- ***Microbiota analysis***

Metagenomic DNA extraction from faecal, and tissue samples and molecular profiling/16S rRNA sequencing will be carried out to explore differences in the microbial community structure between experimental groups. We will examine

the differences in microbial richness (e.g., Chao1) and diversity (e.g., Shannon). ***NB: The tissue collection will be achieved together with other experiments, in order to minimise unnecessary culling and animal use, so no extra animals will be used for this outcome measure.**

- ***CNS phenotype***

To study the gut-brain axis and test whether (epi-)genetic manipulations in ENS cells have an impact on brain function. For this, we will use a combination of non-invasive well-established behavioural test models, such as 2-Object novel object recognition, fear conditioning, object location memory task, maze spontaneous alternation test ***– 12 animals per group***. **No adverse effects are expected from behavioural tests**, but they can cause mild and transient distress that will be closely monitored and attenuated. If any signs of distress appear, animals will be removed immediately from the studies and managed in consultation with the veterinarian. **NB: No need for euthanasia and these experiments can be performed together with other procedures that does not cause greater than mild pain, suffering and distress.**

- ***Development/progression/resolution of infection***

For **experiments using the worm infection model,** we will closely track worm burden, numbers and eggs at different time points in vivo. **NB: No need for euthanasia, so these experiments will be performed together with the ex vivo analysis of tissue and faeces under "Inflammatory processes and immunophenotyping" to following the infection and inflammation.**

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

For the quantitative experiments, design has been based on ARRIVE guidelines and sample sizes were set using power analysis https://www.statstodo.com/SSizCorr_Pgm.php. to determine the number of mice needed for each experiment, generally considering a difference between groups of at least 20%, power of 80% and significance level of 5%.
For qualitative experiments, we will use the minimum number of mice to provide an accurate description based on previous publications and on our own experience.

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
1	mouse	In house breeding	(Young) adults	2385* (see below)	Male and female	Wildtype, Mice with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells, Transgenic mice with altered ENS, Specifically modulate ENS activity	

Provide justifications for these choices

Species	Mice are used due to their anatomical, physiological, and genetic similarity to humans as well as their small size, ease of maintenance, short life cycle, and abundant genetic resources.

Origin	Sox10.CreERT2:R26tdTomato from a licensed non-commercial breeder in UK (but currently in house), Sox10.CreERT2:R26-GCaMP6f and *Wnt1.Cre:R26-hM4Di-DREADD* from licensed non-commercial breeders within Europe,NDRG4$^{fl/fl}$;Wnt1.Cre, NSE-Noggn and Hand2$^{fl/+}$;Wnt1.Cre are all in house, CD63 floxed from a licensed non-commercial breeder in USA
Life stages	Young adults (1-10 months) is most apt for optimal results
Number	Based on the literature, on our own and other collaborators experiences, we estimate that we will need a maximum of 12 mice per group. For FACS experiments, we will need max 9 mice per group and 6 mice for RNA experiments. Total for all lines/groups and all outcome measures, this will be a maximum of 954 animals for the worm infection model alone. For a more detailed overview of how this number is built up, we refer to the following Word document: Total number of mice; table – appendix 3. If dysregulation in the immune system, and/or the microbiome and/or ENS target genes arise from the worm infection challenge then animals can be subjected one or more of the follow-up challenges (e.g. intestinal inflammation challenge and alteration in intestinal microbiota challenge), which requires a similar number of outcome measures to be used for these models (**go/no-go moment**). However, we will choose specific models to continue on the other challenges/models, so we will use maximally 2 mouse lines instead of 4 for follow-up challenges, which means a maximum of 477 mice per challenge. If all challenges/models will be a go (=3 extra), this will maximally lead to 1431 (= 3 x 477) more animals. * A total of maximum 2385 (= 954 + 1431) mice will be used in this appendix including a maximum of all go/no-go animals. In case certain phenotypes are observed in experiments using these lines, other mouse strains can also be added to the same experimental set up to analyse more detailed pathways (lines to specifically study ENS activity, EVs, miRNAs - see list below). ***NB - All genotypes that are not going to be used for the procedures described above can be used in appendix one for culturing cells and tissues, to follow the principles of 3Rs.**
Gender	Both male and female mice will be used to minimise any gender-related variations in the experiment.
Genetic alterations	Mice with fluorescently labelled ENS (e.g. Sox10.CreERT2:R26tdTomato, Wnt1.Cre:R26tdTomato, Sox10.CreERT2:R26-GCaMP6f) will be used to investigate the ENS in depth. Specific ENS cells will be labelled and can thereby be easily followed in these models. Furthermore, activity and specific ENS cells can be studied. This will give us more insight into the function and activity of ENS cells and the interactions between these cell types. Mice with fluorescently labelled extracellular vesicles from ENS cells (e.g. CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre) can be used to study the involvement of extracellular vesicles in the interaction between the ENS and it surrounding tissues and systems. Lines designed to specifically modulate ENS activity (e.g. Wnt1.Cre:R26-hM4Di-DREADD). **(**This line can (but not necessarily will) be used in case of appearance of specific phenotypes related to ENS activity); Mice with a knockdown/overexpression of miRNAs in ENS cells (e.g injected with +AAVx:ENS-specific cre) and/or knockout mice for specific miRNAs that are relevant for gastrointestinal function. Sox10.CreERT2:R26tdTomato will be injected with cell-specific cre dependent +AAVx vector (eg. miRNAs in enteric neurons) in case of a strong phenotype related to miRNA manipulation in *in vitro assays.* Alltogether, these models will help us unravel the interface of the adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota, CNS) in health and may lead to possible pathways and/or targets that could be used for treatment in the future.
Strain	(mixed) C57BL/6J (e.g. Sox10.CreERT2, R26-hM4Di-DREADD, R26-GCaMP6f), (mixed) 129S4SvJaeJ (e.g. Wnt1.Cre). The strain of the wildtype mice will be matched to the corresponding transgenic mise used in the procedure.

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

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.

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

For the inflammation, surgical models and worm infection, animals might experience pain. To minimise pain, suffering and distress, we will use appropriate analgesia and anaesthesia that do not affect the normal intestinal homeostasis. Animals will be constantly monitored as further described in their respective program of work for each experimental procedure to immediately detect any signs of discomfort. Any animals that undergo any experimental procedures and/or manipulation will be closely and often monitored. All other challenges are not predicted to cause the animals any great or long-lasting harm.

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

- *In vivo* experiments can cause distress or mild to moderate discomfort to the animals.
- All animals will be frequently monitored for any signs of pain and distress. Changes to normal and provoked behaviour, movement, physical signs such as posture, respiration, skin and coat changes, inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persists for longer than 24 hours. Any mice exhibiting any deviation from the normal health and behaviour will be further monitored and treated.
- Disease and antibiotic models used in this protocol can cause animals to show sudden body weight loss (of 15% of their body weight).
- Inflammation mouse models can develop side effects, such as pain, weight loss, diarrhoea/constipation and anal bleedings (no ulcers with perforation are expected).
- *H. poly* are mouse pathogens and while wildtype mice are able to clear the worm without exhibiting significant clinical problems, mice carrying mutations may be more susceptible to pathology. All efforts will be made to minimise these symptoms, but it will be necessary to keep animals long enough to achieve measurable responses. Mice challenged with *H. poly* will be monitored for the appearance of clinical signs related to gut inflammation, including diarrhoea, blood stools, rectal prolapse, abdominal discomfort, bloating and weight loss. Infected mice will be observed and weighed during the first week of infection and when new mouse strains are used. Some weight loss is expected and this might be aggravated in mice carrying specific genetic mutations.

Explain why these effects may emerge.

- Most of the side effects mentioned above are unlikely in our non-diseased mice models (unless provoked), but can happen due to inflammation and/or progression of disease, and alteration in intestinal microbiota.

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

Animals will be frequently monitored by experienced staff and researchers. Our mouse colonies will be housed and maintained according to the basic guidelines for animal welfare. Working protocols will be adequately adjusted to benefit not only the research but also animal welfare. Experimental procedures/routes of administration, techniques that cause the least pain, suffering, distress and the shortest lasting harm will be preferred. Experimental procedures will be performed by using aseptic/sterile techniques, and drugs such as analgesics, anaesthetics and antibiotics will be administered whenever necessary. Examples of measures that we will use to prevent or minimise harm are extra hydration in case of diarrhea and boosting gels in case of high weight loss. Measures will be taken accordingly in the case any unexpected adverse effects might appear and animals will be immediately euthanized and experiments stopped in the case of unexpected severe signs of pain and discomfort.

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.

- All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persists for longer than 24 hours.
- Any animal will be immediately euthanised if it exhibit signs of suffering that is not transient or treatable and compromises its normal behaviour.
- Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe disomfort and >20% for severe discomfort) that persist for 48 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision.
- Genetically altered mice used in this appendix are not expected to show any deleterious phenotype, but in case they do, they will be immediately killed.
- Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled.
- Inflammation models: Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 3), "Wasting Syndrome" (body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), or opportunist infections, the animals will be immediately euthanised.
- In the case severe discomfort occur in multiple mice during the experimental procedures described abobe, the experiments will be ended and the animals euthanised.
- *H. poly* - Some weight loss is expected but mice carrying other mutations may show more severe responses. Any mice showing weight loss of 15% of starting weight or diarrhoea, bloody stools, rectal prolapse, abdominal discomfort and bloating for more than 24h will be killed.

Indicate the likely incidence.

For the *H. Poly* and other inflammatory/perturbed models (±10-15% incidence). The animals will be well monitored by experienced people, so that we are able to rapidly detect unforeseen adverse effects in early stages, whereby we aim to avoid reaching the humane endpoints and unexpected drop out.

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).

Appendix 3	Non-diseased gut	H. Poly infection model	Inflammation/perturbed models
Discomfort model itself → Cumulative discomfort ↓	**no discomfort**	**mild discomfort**	**moderate discomfort**
ENS structure /function /proliferation - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Gut physiology - Administration of substances: **mild** - Transit/motility assays: **mild** - Killing: **mild**	Mild	Moderate	Severe
Vesicle trafficking - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Microbiota analysis - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Intercellular communication - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Inflammatory processes and immunophenotyping - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
CNS phenotype - Administration of substances: **mild** - Behavioural tests: **mild**	Mild	Mild	Moderate
Development/progression of worm infection - Administration of substances: **mild** - In vivo tracking: **mild**	Mild	Mild	Moderate

Figure 3: Cumulative discomfort for disease models (columns) combined with outcome measurements (rows). The different mouse lines itself are not expected to have any harmful phenotype or display signs of discomfort.

Approximate percentages expected cumulative discomfort based on animal numbers per outcome measure and disease model:
Mild: 65%
Moderate: 28%
Severe: 7%

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	To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models, for instance the ones described in this document (such as H. poly infection models). In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied *in vivo*. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established *in vitro* culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis *in vivo.* We are also taking advantage of recently established collaborations in the gastroenterology clinic (5.1 lid2h) to obtain human intestinal specimens (IBDs and IBS) and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study disease development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Reduction	Before embarking on any procedures in animal research, we are collecting as many evidence as it is necessary to determine whether a candidate gene or molecule also provide insights on *in vivo* pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This is also can be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals. This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.
Refinement	Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no clear phenotype and results, experiment that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached. Discomfort level in inflammatory, *H. poly* models might be mild to moderate (DSS). In the case of severe discomfort, mice will be immediately sacrificed.

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.

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.

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.

NA

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.

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.

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

Ex vivo (post-mortem) and *in vitro* experiments will be performed in tissue and cells collected from our experimental groups to further analyse the biology of the nervous system.

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.

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

☒ No > Describe the method of killing.

Cervical dislocation

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

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

Appendix 3	Before Go/No-go moment					Maximum numbers after Go/No-go	
Non-diseased gut	***Sox10CreERT2/wnt1Cre-TdTomato (ctrl worm infection)***	***Sox10CreERT2-R26-GCaMP6f (ctrl worm infection)***	***CD63-Sox10CreERT2/wnt1Cre (ctrl worm infection)***	***Sox10CreERT2/wnt1Cre-R26-hM4Di-DREADD (ctrl worm infection)***	***Total***	***Max animals after Go/No-go (ctrl follow-up challenges) Further explained in appendix 3****	***Total***
ENS structure /function /proliferation	#/measure	#/measure	#/measure	#/measure		#/measure	
Number/morphology (a)	12	12	12	12		72	
Activity (b)	12	12	12	12		72	
Proliferation/survival (c)	12	12	12	12		72	
FACS (d)	9	9	9	9	180	54	270
Intercellular communication	#/measure	#/measure	#/measure	#/measure		#/measure	
RNA/ protein	6		6	6		27	
FACS	9		9	9	45	40	67
Gut physiology	#/measure	#/measure	#/measure	#/measure		#/measure	
Whole-gut transit + colonic (a,c)	12		12	12		54	
Small intestinal transit + ex vivo motiliy (b,d)	12		12	12		54	
Permeability (e)	12		12	12	108	54	162
Vesicle trafficking	#/measure	#/measure	#/measure	#/measure		#/measure	
Imaging (a,b,c,d)	12		12	12	36	54	54
Inflammatory processes and immunophenotyping	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Faecal + tissue analysis	36		36	36	108	162	162
Microbiota analysis	#/measure	#/measure	#/measure	#/measure		#/measure	
Faecal + tissue analysis	With other		With other	With other	0	With other	0
CNS phenoytpe	#/measure	#/measure	#/measure	#/measure		#/measure	
Behavioural task 1	With other		With other	With other		With other	
Behavioural task 2	With other		With other	With other		With other	
Behavioural task 3	With other		With other	With other	0	With other	0
Development/progression of worm infection	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Worm burden/number/eggs	With other		With other	With other		With other	
					0		0
*** See figure 2 for Go/No-go decisions. Animals will be subjected to 1 or more challenges following worm infection. Animal models: to be determined.**					477		715

Appendix 3	Before Go/No-go moment					Maximum numbers after Go/No-go	
Diseased gut	***Sox10CreERT2/wnt1Cre-TdTomato (worm infection)***	***Sox10CreERT2-R26-GCaMP6f (worm infection)***	***CD63-Sox10CreERT2/wnt1Cre (worm infection)***	***Sox10CreERT2/wnt1Cre-R26-hM4Di-DREADD (worm infection)***	***Total***	***Max animals after Go/No-go (follow-up challenges) Further explained in appendix 3****	***Total***
ENS structure /function /proliferation	#/measure	#/measure	#/measure	#/measure		#/measure	
Number/morphology (a)	12	12	12	12		72	
Activity (b)	12	12	12	12		72	
Proliferation/survival (c)	12	12	12	12		72	
FACS (e)	9	9	9	9	180	54	270
Intercellular communication	#/measure	#/measure	#/measure	#/measure		#/measure	
Histology/ RNA/ protein	6		6	6		27	
FACS	9		9	9	45	41	68
Gut physiology	#/measure	#/measure	#/measure	#/measure		#/measure	
Whole-gut transit + colonic (a,c)	12		12	12		54	
Small intestinal transit + ex vivo motility (b,d)	12		12	12		54	
Permeability (e)	12		12	12	108	54	162
Vesicle trafficking	#/measure	#/measure	#/measure	#/measure		#/measure	
Imaging (a,b,c,d)	12		12	12	36	54	54
Inflammatory processes and immunophenotyping	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Faecal + tissue analysis	36		36	36	108	162	162
Microbiota analysis	#/measure	#/measure	#/measure	#/measure		#/measure	
Faecal + tissue analysis	With other		With other	With other	0	With other	0
CNS phenoytpe	#/measure	#/measure	#/measure	#/measure		#/measure	
Behavioural task 1	With other		With other	With other		With other	
Behavioural task 2	With other		With other	With other		With other	
Behavioural task 3	With other		With other	With other	0	With other	0
Development/progression of worm infection	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Worm burden/number/eggs	With other		With other	With other		With other	
					0		0
*** See figure 2 for Go/No-go decisions. Animals will be subjected to 1 or more challenges following worm infection. Animal models: to be determined.**					477		716
Total animal number					2385		

NIET-TECHNISCHE PROJECTSAMENVATTING

Naam van het project	Communicatie tussen het enterisch zenuwstelsel, darm lumen en andere weefselomgevingen: van homeostase tot de ontwikkeling en progressie van verscheidene ziektes.
NTS-identificatiecode	NTS-NL-316007 v.1
Nationale identificatiecode van de NTS *Veld wordt niet gepubliceerd.*	
Land	Nederland
Taal	nl
Indiening bij EU *Veld wordt niet gepubliceerd.*	ja
Duur van het project, uitgedrukt in maanden.	60
Trefwoorden	enteric nervous system gastrointestinal tract crosstalk intestinal cancer cells epigenetics
Doel(en) van het project	Fundamenteel onderzoek: Oncologie Fundamenteel onderzoek: Zenuwstelsel Fundamenteel onderzoek: Gastro-intestinaal stelsel met inbegrip van de lever

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).	Het algemene doel van dit project is de organisatie en functie van het enterisch zenuwstelsel (EZS) tijdens ziekte en gezondheid te doorgronden en de interacties van dit buikbrein met andere organen en cellulaire systemen beter te begrijpen. Meer specifiek beogen we om met behulp van nieuw modellen en reeds bestaande methodiek de communicatie tussen het EZS en andere celtypes te bestuderen, de cellulaire en moleculaire interacties in het EZS en tussen het EZS en andere cellulaire systemen te onderzoeken, en de neurale mechanismen die de normale en verstoorde (vb. darmontsteking, darmkanker) werking van het spijsverteringsstelsel reguleren bloot te leggen.
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 het project) en voordelen op lange termijn (die mogelijk pas worden bereikt nadat het project is afgerond).	De complexiteit en locatie van het EZS maken van het onderzoek naar de organisatie en werking van dit buikbrein een hele uitdaging. Echter, dankzij samenwerkingen met andere laboratoria heeft onze onderzoeksgroep de nodige kennis en methodologie om de cellulaire en moleculaire interacties in het EZS en tussen het EZS en andere cellulaire systemen in detail te onderzoeken. Door deze complexiteit en het groot aantal cellulaire systemen waarmee het EZS zijn activiteit integreert zullen de bevindingen van onze studies ook als basis kunnen dienen voor onderzoek naar andere orgaansystemen en het centraal zenuwstelsel. Huidig onderzoek toont ook aan dat de darm, en meer bepaald het EZS als een toegangsdeur kan optreden in het ontstaan van verschillende ziektes die niet gecatalogeerd staan als aandoeningen van het gastro-intestinaal stelsel. Hiertoe behoren onder andere neurodegeneratieve en mentale aandoeningen van het centraal zenuwstelsel. Een beter begrip van de organisatie en functie van het EZS is uiteraard ook essentieel om de ontstaansmechanismen van een hele lijst van gastro-intestinale aandoeningen (enterische neuropathieën, functionele gastro-intestinale aandoeningen (vb. prikkelbaar darmsyndroom) en inflammatoire darmziektes (vb. de ziekte van Crohn) in detail te ontrafelen. Bovendien is er toegenomen evidentie, waaronder recent onderzoek van ons laboratorium, dat aantoont dat het EZS een rol speelt bij de ontwikkeling en progressie van dikke darmkanker. Met onze onderzoeksstrategie, gecombineerd met klinische inzichten van gastro-enterologen, trachten we cellulaire en moleculaire mechanismen te identificeren, en nieuwe biomerkers te vinden, waarmee het ontstaan en de progressie van ziekte kan worden verklaard. Verder zal ons onderzoek duidelijk

	maken in hoeverre en op welke wijze het EZS als therapeutisch doelwit kan worden gebruikt voor de hierboven aangehaalde ziektes.

VOORSPELDE SCHADE

In welke procedures worden de dieren gewoonlijk gebruikt (bijvoorbeeld injecties, chirurgische procedures)? Vermeld het aantal en de duur van deze procedures.	De muizen zullen onderworpen worden aan enkele specifieke procedures. Voor het in vitro deel van het onderzoek zullen de dieren geen specifieke procedures ondergaan, vooraleer we de darm gaan isoleren om onze cel kweek systemen op te zetten. Verder zullen we bepaalde ziektebeelden opwekken in de muizen. Dit zullen we op verschillende manieren aanpakken. We focussen daarbij op het nabootsen van dikke darmkanker en ontstekings-gerelateerde darmkanker. Deze ziektes kunnen we imiteren door bijv. een fout in het genetisch materiaal van de muizen te introduceren of door de muizen te injecteren met een kankerverwekkende stof, al dan niet in combinatie met een ontsteking verwekkende substantie. Als het verkrijgen en analyseren van gegevens met behulp van dit model hier aanleiding toe geven, kunnen dieren ook worden onderworpen aan chemotherapie en radiotherapie. Daarnaast zullen we ons focussen op een darminfectie en afhankelijk van deze resultaten eventueel andere ontstekings-gerelateerde ziektebeelden nabootsen. De modellen zullen vervolgens onderworpen worden aan enkele in vivo analyses (vb het bestuderen van darmfunctie/motiliteit), maar voornamelijk aan ex vivo analyses (vb. het bestuderen van de structuur van het EZS, de ontwikkeling en progressie van kanker/ontsteking). Belangrijk te vermelden is dat we telkens de procedure uitvoeren die een zo minimaal en kort mogelijk effect heeft op pijn/ongemak van de dieren.
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?	We verwachten dat het grootste deel van de muizen geen tot mild ongerief ondervinden: als gevolg van hun genetische afwijkingen in het enterisch zenuwstelsel, kunnen de muizen mogelijk last ondervinden van een veranderde darmmotiliteit. Wanneer de dieren onderworpen worden aan chirurgische procedures, procedures om stoffen te injecteren en/of de verscheidene ziektebeelden na te bootsen, zullen zij een mild tot matig ongerief kunnen ondervinden. Dit voornamelijk als gevolg van een verstoorde darmbeweging, ontstekingen, bloedverlies en gewichtsverlies. Tot slot kan er ook ernstig ongerief optreden op het ogenblik dat de muizen met een veranderd EZS onderworpen worden aan bepaalde ziektemodellen en behandeld worden met radio/chemotherapie, alsook wanneer ze gebruikt worden om de tumor-burden live te volgen dmv CT scans.
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)	5825	0	3942	1343	540

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*

Geef de redenen voor het geplande lot van de dieren na de procedure.	Voor het beoordelen van verschillende darmfunctionaliteiten (bv. darmbeweging/ darmdoorlaatbaarheid) worden dezelfde dieren op verschillende tijdstippen onderworpen aan verschillende experimenten. Ook wanneer de tumor burden bepaald wordt, zullen dezelfde dieren op verschillende tijdstippen CT scans ondergaan. Wanneer de dieren alle experimentele procedures volledig doorlopen hebben, zullen ze gedood worden en zal het materiaal post-mortem gebruikt worden.

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.	Hoewel we de muismodellen nodig hebben om de exacte genetische en fysiologische pathways die belangrijk zijn voor darmkanker, darminfectie, darmontstekingen te ontrafelen, aangezien deze modellen de volledige fysiologie van het lichaam in acht nemen; willen we met dit PV ook verscheidene celkweek systemen opzetten als alternatieve methode. Ons lab heeft ondertussen veel ervaring met het opzetten van deze celkweekmodellen. Dit laat toe om hierin kandidaatgenen/pathways te bestuderen, vooraleer we dit verder onderzoeken a.d.h.v. muizen. Daarnaast hebben we recentelijk ook nieuwe samenwerkingen in het 5.1 lid2h opgezet (vb gastroenterology afdeling), waardoor we ook adhv humaan materiaal reeds de betrokkenheid van het ENS in deze verschillende ziektebeelden kunnen onderzoeken. Echter kunnen we niet volledig afstappen van diergebruik, aangezien deze systemen de volledige complexiteit van het lichamelijk functioneren omvatten. Echter zullen we de meest optimale modellen gebruiken.
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.	Vooraleer we beginnen aan onze dierexperimenten, zullen we informatie verzamelen a.d.h.v. celkweek, (publieke) data en in silico analyses, zodat we gerichtere experimenten kunnen opzetten. We zullen ook de meest optimale modellen gebruiken, waardoor we de hoeveelheid muizen kunnen verminderen. Indien de muizen niet het juiste fenotype vertonen of de eerste experimenten niet gehoopte resultaten opleveren, zullen we niet verder gaan met deze modellen. Omdat we over verschillende onderzoeksprojecten gelijkaardige modellen en procedures gebruiken, kunnen we data interpoleren over de verschillende projecten, waardoor we ook minder dieren nodig hebben. We zullen proberen zoveel mogelijk van onze lijnen als een homozygote lijn aan te houden, waardoor we overbodige kweken en een onnodig overschot van nakomelingen vermijden. Indien dit niet mogelijk is, zullen we onze fokstrategie zo zorgvuldig mogelijk opzetten zodat we heterozygote/homozygote en wildtype nestgenoten (met zelfde leeftijd) kunnen gebruiken voor de experimenten. Indien er onvoldoende informatie beschikbaar is, zullen we 'pilot-experimenten' opzetten, met een beperkt aantal dieren, zodat onze experimentele settings geoptimaliseerd zijn.
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 nieuwe verfijningstechnieken in gebruik te nemen.	De gekozen modellen en procedures zijn fundamenteel om nieuwe inzichten in humane pathologieen die het EZS aantasten. Door modellen te gebruiken waarbij veranderingen in het EZS op een tijdelijke en/of heel specifieke manier geïntroduceerd worden, zullen de bijwerkingen beperkt blijven. Alle protocollen, modellen en behandelingen die onder deze licentie worden gebruikt, zijn standaard en werden eerder en uitgebreid uitgevoerd door leden van ons laboratorium, medewerkers en/of goed gedocumenteerd in relevante literatuur. Pilotstudies zullen ook vooraf worden gescreend om een minimale dosis en blootstellingstijd te verkrijgen die effectief zal zijn met minimaal potentieel lijden. Hierdoor zullen we ervoor zorgen dat de dieren zo weinig mogelijk bijwerkingen / stress ondervinden van de procedures: we zullen dus de nodige analgesie en anesthesie toedienen (die geen effect heeft op het darm functioneren).
Licht de keuze van de soorten en de bijbehorende levensstadia toe	In dit onderzoeksproject is gekozen voor muizen vanwege zowel de anatomische, fysiologische, en genetische gelijkenis met mensen, als de kleine grootte, het gemak van huisvesting en onderhoud, korte levenscyclus, en de grote beschikbaarheid en toegankelijkheid van genetische modellen. Bovendien zijn er veelvuldig toegepaste en valide onderzoeksprotocollen voor het werken met cellen en weefsels van deze dieren en voor het werken met levende dieren. Er zal voornamelijk met volwassen dieren gewerkt worden, vanwege de beschikbaarheid van valide protocollen voor deze leeftijdscategorie voor onze onderzoeksdoelen, en vanwege de betere vertaalbaarheid naar de

	menselijke situatie. Driedimensionale celkweek modellen van het enterisch zenuwstelsel zullen verkregen worden uit muizenembryo's of pasgeborenen, zoals veelvuldig toegepast is in eerdere studies. De driedimensionale celkweek modellen kunnen langdurig gebruikt worden, waardoor er maar een beperkt aantal embryo's of pasgeborenen nodig zal zijn.

VOOR EEN BEOORDELING ACHTERAF GESELECTEERD PROJECT

Project geselecteerd voor BA?	nee
Termijn voor BA	
Reden voor de beoordeling achteraf	
Bevat ernstige procedures	
Maakt gebruik van niet-menselijke primaten	
Andere reden	
Toelichting van de andere reden voor de beoordeling achteraf	

AANVULLENDE VELDEN

Nationaal veld 1 *Veld wordt niet gepubliceerd.*	
Nationaal veld 2 *Veld wordt niet gepubliceerd.*	
Nationaal veld 3 *Veld wordt niet gepubliceerd.*	
Nationaal veld 4 *Veld wordt niet gepubliceerd.*	
Nationaal veld 5 *Veld wordt niet gepubliceerd.*	
Startdatum project *Veld wordt niet gepubliceerd.*	
Einddatum project *Veld wordt niet gepubliceerd.*	
Goedkeuringsdatum project *Veld wordt niet gepubliceerd.*	
ICD-code 1 *Veld wordt niet gepubliceerd.*	
ICD-code 2 *Veld wordt niet gepubliceerd.*	
ICD-code 3 *Veld wordt niet gepubliceerd.*	
Link naar de eerdere versie van de NTS buiten het EC-systeem	

Van: info@zbo-ccd.nl
Verzonden: donderdag 17 februari 2022 14:01
Aan: 5.1 lid2h
Onderwerp: Verzoek om advies over projectvergunningsaanvraag AVD5.1 lid2h202215867
Bijlagen: PV2021_003_5.1 lid2e_Appendix3_IvD.pdf; PV2021_003_5.1 lid2e_NTS_IvD.xlsx; PV2021_003_5.1 lid2e_Projectproposal_IvD.pdf; PV2021_003_5.1 lid2e_Appendix1_IvD.pdf; PV2021_003_5.1 lid2e_Aanvraag_IvD.pdf; PV2021_003_5.1 lid2e_Appendix2_IvD.pdf

Geachte leden van 5.1 lid2h

De Centrale Commissie Dierproeven (hierna: CCD) verzoekt u in het kader van vergunningverlening advies te geven over het project met als titel: "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease " en aanvraagnummer: AVD5.1 lid2h202215867.

Uw commissie wordt verzocht op grond van artikel 10.a.2 van de Wet op de dierproeven de aanvraag te beoordelen en een ethische toetsing uit te voeren waarbij wordt afgewogen of de doelstelling van het project, de verwachte voordelen voor mens, dier of milieu en de haalbaarheid van de doelstellingen, het gebruik van dieren en de schade die zal worden toegebracht aan de dieren in de vorm van lijden, pijn en angst kan rechtvaardigen.

Graag ontvangen wij van u bericht dat deze e-mail goed is ontvangen en wanneer u dit advies in de vergadering gaat bespreken.

Voor het in te dienen advies dient de DEC gebruik te maken van de meest actuele versie van het op de website van de CCD gepubliceerde Format DEC-advies en de toelichting daarbij. U dient deze aanvraag vertrouwelijk te behandelen. Voor de communicatie met de CCD dient u gebruik te maken van FileSecure.

De CCD verzoekt u uiterlijk binnen 20 werkdagen, na 17-02-2022, uw advies bij de CCD in te dienen. Indien de aanvraag door uw commissie niet in behandeling kan worden genomen, dient u dit per ommegaande per e-mail aan de CCD te melden.

Ingeval uw commissie tussentijds aanvullende informatie wil inwinnen bij de aanvrager wordt de termijn opgeschort en geeft u in uw advies aan wanneer dit is geweest. Opschorting van de adviestermijn vindt niet plaats ingeval u ten behoeve van uw advies een onafhankelijk extern expert raadpleegt. Mocht u verwachten door een andere reden dan opschorting uw advies later dan 20 werkdagen na 17-02-2022 bij de CCD in te dienen, dan verzoeken wij u dit direct aan de CCD te melden.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Centrale Commissie Dierproeven

www.centralecommissiedierproeven.nl

.....
Postbus 93118 | 2509 AC | Den Haag
.....

T: 0800 789 0789
E: info@zbo-ccd.nl

5.1 lid2h

Instantie voor Dierenwelzijn

IvD Secretariaat

5.1 lid2h

Aan: CCD

Uw kenmerk	*Ons kenmerk*	*Doorkiesnummer*	5.1 lid2h
	AV2022-004	81006	22-03-2022

Geachte heer, mevrouw,

Bijgesloten treft u een verzoek tot Melding aan die betrekking heeft op het projectvoorstel van PV 2021-003 behorende bij beschikkingsnummer AVD5.1 lid2h202215867.

Dit verzoek (d.d. 22-03-2022) is door de Instantie van Dierenwelzijn-5.1 lid2h behandeld, en op basis van de onderbouwing ervan en het feit dat er geen toename zal zijn in het ongerief of aantallen dieren, acht de 5.1 lid2h dit een aanpassing in het PV die in de categorie 'wijziging PV' valt.

Hoogachtend,

5.1 lid2e

Instantie voor Dierenwelzijn

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*	5.1 lid2h
☐ 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	AVD5.1 lid2h 202215867

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

Naam instelling of organisatie	5.1 lid2h			
Titel, voorletters en achternaam van de portefeuillehouder	Titel	Voorletters	Achternaam	☒ Dhr. ☐ Mw
E-mailadres contactpersoon	5.1 lid2e			
Titel, voorletters en achternaam van de diens gemachtigde (indien van toepassing)	Titel	Voorletters	Achternaam	☐ Dhr. ☐ Mw
E-mailadres gemachtigde				

Vul de gegevens van het postadres in.

Straat en huisnummer	5.1 lid2h
Postcode en plaats	
Postbus, postcode en plaats	

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	5.1 lid2e	☒ Dhr. ☐ Mw.
Functie	5.1 lid2e	
Afdeling	5.1 lid2h	

	Telefoonnummer	5.1 lid2e	
	E-mailadres		
1.5 *(Indien van toepassing)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.	(Titel) Naam en voorletters		☐ Dhr. ☐ Mw.
	Functie		
	Afdeling		
	Telefoonnummer		
	E-mailadres		
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	5.1 lid2h	
	E-mailadres	5.1 lid2h	
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 hier onder 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 geen negatieve gevolgen kan hebben voor het dierenwelzijn?

☐ Nee > Ga verder met vraag 3

☒ Ja > Geef hier onder weer wat deze melding inhoudt en ga verder met vraag 6

Aanpassing van de Verantwoordelijk onderzoeker op het aanvraagformulier, naar 5.1 lid2e

3 Over uw project

3.1 Wat is de geplande start- en einddatum van het project?	Startdatum	01-03-2022
	Einddatum (t/m)	01-03-2027
3.2 Wat is de titel van het project?	The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease	
3.3 Wat is de titel van de niet-technische samenvatting?	Communicatie tussen het enterisch zenuwstelsel, darm lumen en andere weefselomgevingen: van homeostase tot de ontwikkeling en progressie van verscheidene ziektes.	
3.4	Naam DEC	5.1 lid2e
	Postadres	

Wat is de naam van de Dierexperimentencommissie (DEC) van voorkeur?	E-mailadres	5.1 lid2h	

4 Factuurgegevens

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

Naam:		Afdeling:
Straat:		Huisnummer:
Postcode:	Plaats:	
Postbus:	Postcode:	Plaats:
E-mail:		

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

Ordernummer:

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	5.1 lid2e
Functie	
Plaats	5.1 lid2h
Datum	22-03-2022
Handtekening	5.1 lid2e

5.1 lid2h Advies PV 2021-003-AVD5.1 lid2e202215867; 5.1 lid2e

Preambule

De 5.1 lid2h verzoekt U eventuele aanvullende vragen rechtstreeks aan de aanvrager te stellen met een afschrift aan de 5.1 lid2h via een beveiligde verbinding.

A. Algemene gegevens over de procedure

1. **Aanvraagnummer:** 5.1 lid2h

2. **Titel van het project:** The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease

3. **Titel van de NTS:** Communicatie tussen het enterisch zenuwstelsel, darm lumen en andere weefselomgevingen: van homeostase tot de ontwikkeling en progressie van verscheidene ziektes.

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

5. **Contactgegevens DEC:** 5.1 lid2h contactpersoon: 5.1 lid2e , emailadres: 5.1 lid2h

6. **Adviestraject (data dd-mm-jjjj):**
   - ☒ ontvangen door DEC 17.02.2022
   - ☒ aanvraag compleet 17.02.2022
   - ☒ in vergadering besproken 25.02.2022
   - ☐ anderszins behandeld
   - ☒ termijnonderbreking(en)
     a. van 02.03.2022 tot 13.04.2022 (toelichtingsvragen gesteld)
     b. van 19.04.2022 tot 20.05.2022 (toelichtingsvragen gesteld)
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen
   - ☒ aanpassing aanvraag
   - ☒ advies aan CCD 30.05.2022

7. **Geef aan of de aanvraag is afgestemd met de IvD en deze de instemming heeft van de IvD.** De IvD geeft aan dat de aanvrager de aanvraag met de IvD heeft afgestemd en dat deze de instemming heeft van de IvD.

8. **Eventueel horen van aanvrager:** N.v.t.

9. **Correspondentie met de aanvrager:**
   - **Datum:** 02.03.2022 en 19.04.2022
   - **Gestelde vraag/vragen:** Zie bijlage I en II
   - **Datum antwoord:** 13.04.2022 en 20.05.2022
   - **Verstrekt(e) antwoord(en):** Zie bijlage I en II
   - **De antwoorden hebben** wel **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. **Is het project vergunningplichtig (dierproeven in de zin der wet)? Indien van toepassing, licht toe waarom het project niet vergunningplichtig is en of daar discussie over geweest is.** (*Indien niet vergunningplichtig, ga verder met onderdeel E. Advies)***.** JA

2. **De aanvraag betreft** een nieuwe aanvraag

3. **Is de DEC competent om hierover te adviseren?** JA

4. **Geef aan of DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, zijn uitgesloten van de behandeling van de aanvraag en het opstellen van het advies. Indien van toepassing, licht toe waarom.** NEE

C. Beoordeling (inhoud)

1. **Beoordeel of de aanvraag toetsbaar is en voldoende samenhang heeft** *(Zie bijlage I handreiking 'Invulling definitie project volgens de versie 2022'; zie bijlage III voor CCD-toelichting en voorbeeld)***.**

   Deze aanvraag heeft een concrete doelstelling en kan getypeerd worden als een programma. Het programma lijkt de meeste kenmerken te hebben van voorbeeld 4 uit de Handreiking 'Invulling definitie project'. Het is helder welke handelingen en ongerief individuele dieren zullen ondergaan. De 5.1 lid2h vertrouwt erop dat de aanvrager gedurende het programma op zorgvuldige wijze besluiten zal nemen over de voortgang van het programma en er niet onnodig dieren gebruikt zullen worden. Gezien bovenstaande is de 5.1 lid2h van mening dat de aanvraag toetsbaar is en voldoende samenhang heeft.

2. **Signaleer of er mogelijk tegenstrijdige wetgeving is die het uitvoeren van de proef in de weg zou kunnen staan. Het gaat hier om wetgeving die gericht is op de gezondheid en welzijn van het dier of het voortbestaan van de soort (bijvoorbeeld Wet dieren en Wet Natuurbescherming).**

   Voor zover de 5.1 lid2h de mogelijke tegenstrijdigheid kan beoordelen is er geen aanleiding om deze strijdigheid met andere wettelijke bepalingen aanwezig te achten.

3. **Beoordeel of de in de projectaanvraag aangekruiste doelcategorie(ën) aansluit(en) bij de hoofddoelstelling. Nevendoelstellingen van beperkt belang hoeven niet te worden aangekruist in het projectvoorstel.**

   Het voorstel heeft inderdaad kenmerken *van fundamenteel onderzoek*, immers draait het hele onderzoek om het beter begrijpen van de rol van het enterisch zenuwstelsel in het ontstaan en de progressie van pathologische fysiologie in de darm.

Belangen en waarden

4. **Benoem zowel het directe doel als het uiteindelijke doel en geef aan of er een directe en reële relatie is tussen beide doelstellingen. Beoordeel of het directe doel gerechtvaardigd is binnen de context van het onderzoeksveld** *(Zie bijlage II ' Praktische handreiking ETK' : Stap 1.C4; zie bijlage III voor CCD-voorbeeld)***.**

   De directe doelen van het programma zijn:
   Het verkrijgen van inzicht in de mechanismen die ten grondslag liggen aan communicatie tussen verschillende typen (primaire en kanker)cellen en cellen van het enterisch zenuwstelsel, de cellulaire en moleculaire mechanismen die betrokken zijn bij de interactie tussen het enterisch zenuwstelsel en andere typen cellen in een kanker-omgeving en de mechanismen die de communicatie tussen cellen beïnvloeden in homeostase en darmziekten (worminfectie, microbiota-verandering, inflammatie).
   Het uiteindelijke doel is inzicht te krijgen in hoe het enterisch zenuwstelsel betrokken is bij het ontstaan en de verdere ontwikkeling van ziekten van de darm. Daaruit voortvloeiend zouden uiteindelijk nieuwe aanknopingspunten voor behandelingen van darmziekten kunnen worden gevonden. Het betreft hier een fundamenteel programma. Er is binnen dit programma een reële relatie tussen het directe doel en het uiteindelijke doel. De 5.1 lid2h acht het waarschijnlijk dat het uiteindelijke doel deels behaald zal worden binnen de duur van dit programma.

   De aanvrager heeft helder gemaakt wat de status van het onderzoeksveld is en wat de bijdrage van dit project aan het onderzoeksveld zal zijn. Uit de aanvraag blijkt dat de kennis van de cellulaire en moleculaire mechanismen die betrokken zijn bij communicatie tussen diverse typen cellen en het enterisch zenuwstelsel op dit moment zeer beperkt is, dat deze kennis noodzakelijk is voor het ontwikkelen van nieuwe behandelingsmogelijkheden bij een breed scala aan darmaandoeningen en dat daar op dit terrein ook behoefte aan is. De 5.1 lid2h is van mening dat het directe doel, het verkrijgen van inzicht in de mechanismen die ten grondslag liggen aan communicatie tussen verschillende typen (primaire en kanker)cellen en cellen van het enterisch zenuwstelsel, de cellulaire en moleculaire mechanismen die betrokken zijn bij de interactie tussen het enterisch zenuwstelsel en andere typen cellen in een kanker-omgeving en de mechanismen die de communicatie tussen cellen beïnvloeden in homeostase en darmziekten, gerechtvaardigd is binnen de context van het onderzoeksveld, mits ernstig ongerief wordt vermeden.

5. **Benoem de belanghebbenden in het project en beschrijf voor elk van de belanghebbenden welke morele waarden in het geding zijn of bevorderd worden** *(Zie bijlage II 'Praktische handreiking ETK': Stap 2.B en tabel 1; zie bijlage III voor CCD-voorbeeld)***.**

   De belangrijkste belanghebbenden in dit fundamentele programma dat gericht is op het verkrijgen van inzicht in de communicatie tussen het enterisch zenuwstelsel en verschillende celtypen in de darm gedurende homeostase en pathologische omstandigheden zijn de proefdieren, de wetenschappelijke gemeenschap en uiteindelijk patiënten, zorgverleners en de farmaceutische industrie. De laatste drie belanghebbenden zijn nu nog niet direct in beeld, maar worden door de onderzoekers wel expliciet genoemd. Vanwege het fundamentele karakter van dit programma zullen deze drie belanghebbenden bij de ethische overweging verder niet meegenomen worden.

   Waarden die voor proefdieren in het geding zijn:
   De fundamentele waarde van leven zal de dieren in het kader van voorgesteld onderzoek ontnomen worden. Daarnaast zullen de dieren aangetast worden in hun integriteit en mogelijkheid tot uitoefening van soorteigen gedrag door de experimentele

handelingen (injecties (o.a. voor het induceren van genetische veranderingen en het toedienen van chemische middelen), inductie van darmkanker (via injecties, of via colonoscopie), CT-scans met voorafgaand voedseldeprivatie, bestraling, chemotherapie, verschillende manieren voor het induceren van darminflammatie (worminfectie, dextraan natriumsulfaat (DSS)), laparotomie, het veranderen van de darmflora (antibioticumkuur gevolgd door fecaal transplantaat, vasten) en het leven met de gevolgen daarvan, waaronder het ondervinden van ongerief gedurende de proeven.

***Waarden die voor onderzoekers bevorderd worden**:*
De onderzoekers zullen meer kennis verkrijgen over de rol en de werking van het enterisch zenuwstelsel, waardoor ook kennis over mechanismen die al dan niet een rol spelen bij diverse darmaandoeningen zal toenemen. Daardoor zullen de onderzoekers als experts in het veld geïdentificeerd worden, en andere onderzoekers zullen aanknopingspunten voor vervolgonderzoek aangereikt krijgen.

Waarden die voor patiënten bevorderd worden:
Op de lange termijn zullen de patiënten kunnen profiteren van beter inzicht in hun darmaandoeningen. Daardoor zullen mogelijk ook nieuwe aangrijpingspunten voor behandelingen in zicht komen.

Waarden die voor zorgverleners bevorderd worden:
Op de lange termijn kunnen op basis van de verworven inzichten mogelijk nieuwe therapieën ontwikkeld worden wat het behandelingsrepertoire van zorgverleners zal vergroten.

Waarden die voor de farmaceutische industrie bevorderd worden:
Wanneer er nieuwe aanknopingspunten voor behandelingen van darmaandoeningen gevonden worden, zal de farmaceutische industrie mogelijk nieuwe medicijnen kunnen ontwikkelen en op de markt brengen.

6. Is er aanleiding voor de DEC om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken?

Voor zover de 5.1 lid2h de beschreven effecten op het milieu kan beoordelen is er geen aanleiding om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken.

Proefopzet en haalbaarheid

7. Beoordeel of de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven voldoende gewaarborgd zijn. Licht uw beoordeling toe. *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C5).*

Voor zover de 5.1 lid2h kan beoordelen zijn de kennis en kunde van de onderzoeksgroep adequaat gezien de jarenlange ervaring met proefdiermodellen voor het bestuderen van het enterisch zenuwstelsel, wetenschappelijke output, de verworven interne- en externe financiering alsmede de aandacht voor de drie V's onder meer geïllustreerd aan de hand van publicaties in tijdschriften als Nature Reviews Gastroenterology & Hepatology, American Journal of Physiology Gastrointestinal and Liver Physiology, J Neurogastroenterology and Motility en Glia.

8. Beoordeel of het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en of de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project. Licht uw beoordeling toe. *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C6)*.

De 5.1 lid2h is ervan overtuigd dat het projectvoorstel aansluit bij recente wetenschappelijke inzichten aangaande het verkrijgen van inzicht in de mechanismen die ten grondslag liggen aan communicatie tussen verschillende typen (primaire en kanker)cellen en cellen van het enterisch zenuwstelsel, de cellulaire en moleculaire mechanismen die betrokken zijn bij de interactie tussen het enterisch zenuwstelsel en andere typen cellen in een kanker-omgeving en de mechanismen die de communicatie tussen cellen beïnvloeden in homeostase en darmziekten (worminfectie, microbiota-verandering, inflammatie), en geen hiaten bevat die de bruikbaarheid van de resultaten in de weg zullen staan. De voorgestelde experimentele opzet en uitkomstparameters zijn logisch en helder gekozen en sluiten aan bij de aangegeven doelstellingen en de gekozen strategie en experimentele aanpak kunnen naar de mening van de 5.1 lid2h leiden tot het behalen van de doelstelling in het kader van het programma, ook zonder dat een deel van de dieren ernstig ongerief ondergaat.

Welzijn dieren

9. Geef aan of er sprake is van één of meerdere bijzondere categorieën van dieren, omstandigheden of behandeling van de dieren. Beoordeel of de keuze hiervoor voldoende wetenschappelijk is onderbouwd en of de aanvrager voldoet aan de in de Wet op de Dierproeven (Wod). voor de desbetreffende categorie genoemde beperkende voorwaarden. Licht uw beoordeling toe.
(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C1; zie bijlage III voor CCD toelichting en voorbeelden).

- ☐ **Bedreigde diersoort(en) (10e, lid 4)**
- ☐ **Niet-menselijke primaten (10e)**
- ☐ **Dieren in/uit het wild (10f)**
- ☐ **Niet gefokt voor dierproeven (11, bijlage I richtlijn)**
- ☐ **Zwerfdieren (10h)**
- ☐ **Hergebruik (1e, lid 2)**
- ☐ **Locatie: buiten instelling vergunninghouder (10g)**
- ☐ **Geen toepassing verdoving/pijnbestrijding (13)**
- ☐ **Dodingsmethode niet volgens bijlage IV richtlijn (13c, lid 3)**

Niet van toepassing.

10. Geef aan of de dieren gehuisvest en verzorgd worden op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU. Indien niet aan deze minimale eisen kan worden voldaan, omdat het, om redenen van dierenwelzijn of diergezondheid of om wetenschappelijke redenen, noodzakelijk is hiervan af te wijken, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe.

Een deel van de dieren zal maximaal 16 uur zonder voedsel gezet worden, zodat er geen voedsel in de darmen zit bij het uitvoeren van de CT-scans. De 5.1 lid2h is er verder van verzekerd dat voldaan wordt aan huisvesting en verzorging volgens de richtlijn op basis van de daartoe strekkende verklaring (in duplo) van zowel de vertegenwoordiger van de vergunninghouder, als de aanvrager onder respectievelijk punt 6 der ondertekening van de aanvraag en punt F in de bijlagen.

11. Beoordeel of het cumulatieve ongerief als gevolg van de dierproeven voor elk dier realistisch is ingeschat en geclassificeerd. Licht uw beoordeling toe.
(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C2).

De 5.1 lid2h acht het ongerief grotendeels realistisch ingeschat. In het voorstel wordt aangegeven dat in totaal 68% van de dieren licht, 23% matig en 9% ernstig ongerief

zal kunnen ervaren. De 5.1 lid2h vindt dat ernstig ongerief binnen deze aanvraag niet gerechtvaardigd is. Uit de antwoorden van de onderzoekers (vraag 4, tweede ronde) is namelijk gebleken dat er op een lopende vergunning pilotstudies uitgevoerd zullen worden waarbij de optimale omstandigheden voor de experimenten met potentieel ernstig ongerief bepaald worden. Wanneer de uitkomst van de pilotstudies is dat er ernstig ongerief zal optreden, is dit aangemerkt als een 'no-go' voor de betreffende vervolg-experimenten binnen deze aanvraag.

12. **Het uitvoeren van dierproeven zal naast het ongerief vaak gepaard gaan met aantasting van de integriteit van het dier. Beschrijf op welke wijze er sprake is van aantasting van integriteit.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C2). (zie bijlage III voor CCD-voorbeeld).*

    De integriteit van de dieren zal worden aangetast door de experimentele handelingen (injecties (o.a. voor het induceren van genetische veranderingen en het toedienen van chemische middelen), inductie van darmkanker (via injecties, of via colonoscopie), CT-scans voorafgegaan door voedseldeprivatie, bestraling, chemotherapie, verschillende manieren voor het induceren van darminflammatie (worminfectie, dextraan natriumsulfaat (DSS)), laparotomie, het veranderen van de darmflora (antibioticumkuur gevolgd door fecaal transplantaat, vasten) en het leven met de gevolgen daarvan, de dieren zullen beperkt worden in hun natuurlijke gedrag en gedurende de proeven zullen de dieren stress ondervinden. De integriteit van dieren wordt tevens fysiek aangetast door de (darmen van de) dieren genetisch te veranderen.

13. **Beoordeel of de criteria voor humane eindpunten goed zijn gedefinieerd en of goed is ingeschat welk percentage dieren naar verwachting een humaan eindpunt zal bereiken. Licht uw beoordeling toe.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C3)*.

    Naar de mening van de 5.1 lid2h zijn de humane eindpunten zorgvuldig beschreven en is de inschatting van het percentage van 10-15% van de dieren bij de inflammatiemodellen, colorectaalkankermodellen en dieren die chemotherapie en bestraling krijgen dat naar verwachting een humaan eindpunt zal bereiken eveneens zorgvuldig beschreven in de projectaanvraag.

3V's

14. **Beoordeel of de aanvrager voldoende aannemelijk heeft gemaakt dat er geen geschikte vervangingsalternatieven zijn. Licht uw beoordeling toe.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C3)*.

    De 5.1 lid2h is van mening dat de doelstellingen van de proef niet behaald kunnen worden, anders dan met de aangevraagde dieren, daar geschikte vervangingsalternatieven reeds worden ingezet – zoals in vitro experimenten met menselijke cellen – waar mogelijk, zoals beschreven in onderhavig projectvoorstel.

15. **Beoordeel of het aantal te gebruiken dieren realistisch is ingeschat en of er een heldere strategie is om ervoor te zorgen dat tijdens het project met zo min mogelijk dieren wordt gewerkt waarmee een betrouwbaar resultaat kan worden verkregen. Licht uw beoordeling toe.** *(Zie Praktische handreiking ETK: Stap 1.C3)*.

    Naar de mening van de 5.1 lid2h is het aantal te gebruiken dieren realistisch ingeschat en wel zodanig dat niet meer dan nodig, maar ook niet minder dan nodig, dieren worden gebruikt voor het behalen van een betrouwbaar wetenschappelijk resultaat zulks gebaseerd op de eigen ervaring van de onderzoekers met dit type experimenten en

wetenschappelijke literatuur.

16. Beoordeel of het project in overeenstemming is met de vereiste van verfijning van dierproeven en het project zodanig is opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Licht uw beoordeling toe. *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C3)*.

De 5.1 lid2h heeft zich ervan verzekerd dat de aanvrager al het mogelijke zal doen om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen zonder dat dit het behalen van de doelstelling in de weg staat. Hierbij heeft de 5.1 lid2h onder andere de pijnbestrijding, voeding en huisvesting in haar beoordeling betrokken.

17. Beoordeel, indien het wettelijk vereist onderzoek betreft, of voldoende aannemelijk is gemaakt dat er geen duplicatie plaats zal vinden en of de aanvrager beschikt over voldoende expertise en informatie om tijdens de uitvoering van het project te voorkomen dat onnodige duplicatie plaatsvindt. Licht uw beoordeling toe.

Het betreft hier geen wettelijk vereist onderzoek.

Dieren in voorraad gedood en bestemming dieren na afloop proef

18. Geef aan of dieren van beide geslachten in gelijke mate ingezet zullen worden. Indien alleen dieren van één geslacht gebruikt worden, beoordeel of de aanvrager dat in voldoende mate wetenschappelijk heeft onderbouwd. *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C3; zie bijlage III voor CCD-voorbeeld).*

De aanvrager zal in het project gebruik maken van zowel mannelijke als vrouwelijke dieren.

19. Geef aan of dieren gedood worden in kader van het project (tijdens of na afloop van de dierproef). Indien dieren gedood worden, geef aan of en waarom dit noodzakelijk is voor het behalen van de doelstellingen van het project. Indien dieren gedood worden, geef aan of er een voor de diersoort passende dodingsmethode gebruikt wordt die vermeld staat in bijlage IV van richtlijn 2010/63/EU. Zo niet, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe. Indien van toepassing, geeft ook aan of er door de aanvrager ontheffing is aangevraagd. *(Zie bijlage II 'Praktische handreiking ETK': Stap 1.C3).*

Naar de mening van de 5.1 lid2h is dit genoegzaam beschreven in de projectaanvraag door de aanvrager, want de dieren worden gedood in het kader van de proef (isoleren cellen, weefsels, enzovoorts).

20. Indien dieren worden gedood om niet-wetenschappelijke redenen, is herplaatsing of hergebruik overwogen? Licht toe waarom dit wel/niet mogelijk is. Niet van toepassing.

NTS

21. Is de niet-technische samenvatting een evenwichtige weergave van het project en begrijpelijk geformuleerd?

Naar de mening van de 5.1 lid2h is zulks het geval.

D. Ethische afweging

1. **Benoem de centrale morele vraag.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 3.A).*

   Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan?

2. **Weeg voor de verschillende belanghebbenden, zoals beschreven onder C5, de sociale en morele waarden waaraan tegemoet gekomen wordt of die juist in het geding zijn, ten opzichte van elkaar af. Om dit proces te vergemakkelijken, kunt u de belangrijkste belanghebbenden en de belangrijkste waarden die in het geding zijn waarderen. U kunt dit verwoorden in termen van gering, matig of veel/ernstig voordeel of nadeel. Geef aan waarom de DEC bevordering van waarden (baten) voor de ene belanghebbende prevaleert boven de aantasting van waarden (kosten) voor de andere belanghebbende.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 3.B; zie bijlage III voor CCD-voorbeelden).*

   - Waarden die voor de proefdieren in het geding zijn: substantieel nadeel
   - Waarden die voor onderzoekers bevorderd worden: reëel voordeel.
   - Waarden die voor de medische wetenschap bevorderd worden: reëel voordeel.

   De 5.1 lid2h is van mening dat de belangen van onderzoekers/wetenschappelijke instituten, binnen het project 'The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease' zwaarder wegen dan de belangen/waarden van de proefdieren/dierbeschermingsorganisaties, mits ernstig ongerief wordt voorkomen. Voor de betrokken proefdieren leiden de beschreven proeven tot de dood na, in het voorstel aangegeven licht ongerief voor 68% van de dieren, matig ongerief voor 23% van de dieren en ernstig ongerief voor 9% van de dieren. De 5.1 lid2h is echter van mening dat ernstig ongerief voorkomen dient te worden, omdat er een go-no go is ingebouwd door de onderzoekers tussen deze aanvraag en een reeds lopende vergunning. Wanneer uit de pilotstudies onder de lopende vergunning blijkt dat ernstig ongerief niet vermeden kan worden, zullen de betreffende experimenten in de huidige aanvraag geen doorgang vinden (zie antwoord op vraag 4 uit de tweede ronde vragen).

   De dieren worden door de experimenten in hun welzijn geschaad. De integriteit van de dieren zal worden aangetast door de experimentele handelingen (injecties (o.a. voor het induceren van genetische veranderingen en het toedienen van chemische middelen), inductie van darmkanker (via injecties, of via colonoscopie), CT-scans (met bijbehorende voedseldeprivatie), bestraling, chemotherapie, verschillende manieren voor het induceren van darminflammatie (worminfectie, dextraan natriumsulfaat (DSS)), laparotomie, het veranderen van de darmflora (antibioticumkuur gevolgd door fecaal transplantaat, vasten) en het leven met de gevolgen daarvan gedurende de proeven en de opoffering aan het eind daarvan.

   Indien de doelstellingen bereikt worden, zal dit project echter leiden tot meer inzicht in de communicatie tussen het enterische zenuwstelsel en andere celtypen in homeostase of onder pathologische omstandigheden zoals inflammatie, kanker of veranderingen in de microbiota. Daardoor zullen aandoeningen aan de darm mogelijk beter begrepen worden en worden deuren geopend naar nieuwe interventies.

Het is aannemelijk dat de doelstelling behaald zal worden. De onderzoekers zullen zoveel mogelijk trachten het lijden van de dieren te beperken.

3. **Beantwoord de centrale morele vraag. Maak voor het beantwoorden van deze vraag gebruik van bovenstaande afweging van morele waarden. Maak daarnaast gebruik van de volgende moreel relevante feiten: belang onderzoek (C4), kennis en kunde van betrokkenen (C7), haalbaarheid doelstellingen (C8), categorieën en herkomst dieren (C9), 3V's (C14-C18), ongerief (C10-13 en C19) en relevante wet en regelgeving (C2). Onderbouw hoe al deze elementen zijn meegewogen bij de beantwoording van de centrale morele vraag, zodanig dat het navolgbaar is zonder gedetailleerde kennis te hebben van het projectvoorstel.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 3.C; zie bijlage III voor CCD-voorbeeld)*.

   De 5.1 lid2h beantwoordt de centrale morele vraag 'Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan?' bevestigend.

   De 5.1 lid2h onderschrijft de integriteit en intrinsieke waarde van het dier en heeft oog voor het te ondergane ongerief van de proefdieren. Naar haar mening weegt het reële belang van dit project, en meer specifiek de belangen van de onderzoekers en het betreffende onderzoeksgebied zwaarder dan de voorgestelde schending van integriteit, het te berokkenen ongerief en opoffering, mits ernstig ongerief wordt vermeden.

   De 5.1 lid2h is van mening dat de voorgestelde experimentele opzet en de uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en dat de gekozen strategie en de voorgestelde experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het programma. De onderzoekers beschikken over de benodigde kennis en technische expertise, zoals duidelijk uit hun voorstel blijkt. Er is geen sprake van duplicatie.

   In de gekozen strategie wordt op bevredigende wijze tegemoetgekomen aan de vereisten van vervanging, vermindering en verfijning. De 5.1 lid2h is ervan overtuigd dat de aanvrager voldoende maatregelen treft om zowel het ongerief van de dieren als het aantal benodigde dieren tot een minimum te beperken. Er zijn voldoende *go/no-go* momenten voorzien om onnodige dierproeven te vermijden. De 5.1 lid2h is ervan overtuigd dat er geen alternatieven zijn, waardoor deze dierproef met minder ongerief of met minder, dan wel zonder levende dieren zou kunnen worden uitgevoerd. Wel is zij van mening dat ernstig ongerief vermeden kan en moet worden.

   Op grond van deze overwegingen beschouwt de 5.1 lid2h de voorgestelde dierproeven in het projectvoorstel "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" als ethisch gerechtvaardigd, mits ernstig ongerief wordt vermeden. Derhalve voorziet de 5.1 lid2h het projectvoorstel "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" van een positief advies onder voorwaarde dat ernstig ongerief voor de dieren vermeden zal worden.

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:*** Dat de dieren niet meer dan matig ongerief zullen ondervinden. De onderzoekers geven aan dat uitkomsten van pilotstudies op een lopende vergunning zorgen voor een go-no go voor het deel van de experimenten die hier worden aangevraagd. Daarbij is een no-go van toepassing wanneer uit de pilot-experimenten blijkt dat ernstig ongerief niet vermeden kan worden.
   - ☐ **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 kan unaniem tot stand zijn gekomen dan wel gebaseerd zijn op een meerderheidsstandpunt in de DEC. Indien gebaseerd op een meerderheidsstandpunt, specificeer het minderheidsstandpunt op het niveau van verschillende belanghebbenden en de waarden die in het geding zijn.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 4.A; zie bijlage III voor CCD-voorbeeld).*

   Het uitgebrachte 5.1 lid2h advies is tot stand gekomen op basis van een meerderheidsstandpunt. Een DEC-lid nam een minderheidsstandpunt in. De overwegingen hiervoor waren: (1) het grote aantal muizen staat nauwelijks in verhouding tot de praktische baten van het onderzoek; aangezien het fundamenteel onderzoek betreft is het onzeker of hier nieuwe therapieën uit voort gaan komen; (2) het gebruik van genetisch gemodificeerde muizen.

3. **Omschrijf de knelpunten/dilemma's die naar voren zijn gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies zowel binnen als buiten de context van het project.** *(Zie bijlage II 'Praktische handreiking ETK': Stap 4.B)*.

   Het feit dat het een fundamenteel onderzoek betreft waarin 1. duizenden dieren worden aangevraagd waarvan 2. een deel maximaal ernstig ongerief kan ondergaan zijn belangrijke discussiepunten geweest. Ook zijn er extra vragen gesteld over pijnstilling en pilotexperimenten die nodig zijn voor een deel van de experimenten die onder Bijlage 2 beschreven staan. Daarnaast heeft de 5.1 lid2h zich afgevraagd of het programma binnen de gestelde tijdsduur realistisch is: dus of het project haalbaar is.

Bijlage I:

Vragen 5.1 lid2h d.d. 02.03.2022 en Antwoorden VO d.d. 13.04.2022

Bijlage I. Fundamentele vragen 5.1 lid2h

Algemeen

1. Waarom weegt voor u(w onderzoeksgroep) het belang van het voorgestelde project zwaarder dan het matige ongerief en de schending van integriteit en de beëindiging van het leven dat de proefdieren wordt aangedaan?

Het doel van dit project is het in kaart brengen en begrijpen van de organisatie en functie van het enterisch zenuwstelsel (enteric nervous system, ENS) en van de interacties die het aangaat met andere cellulaire systemen, tijdens zowel fysiologische als pathologische condities. Wij achten dit van groot belang omdat we met het uitvoeren van dit project een beter inzicht krijgen in de rol van het ENS in de initiatie en progressie van gastro-intestinale ziektes en de mechanismen die hierbij betrokken zijn. Het staat vast dat het ENS een belangrijke regulator van darm homeostase en pathologie is. Echter, de details omtrent de moleculaire, cellulaire en (patho-)fysiologische controlemechanismen zijn veelal niet begrepen. De kennis die wordt verkregen uit dit onderzoek zal ervoor zorgen dat wij als onderzoeksgroep grote stappen vooruit kunnen maken binnen ons onderzoeksgebied. Daarnaast zullen andere onderzoeksgroepen hun voordeel kunnen doen met deze kennis en zullen de ontdekkingen die gedaan worden binnen dit project kunnen bijdragen aan de ontwikkeling van nieuwe targets en therapieën voor deze ziekte beelden. Wij zijn dus van mening dat de bovengenoemde voordelen opwegen tegen de nadelen van dit onderzoek, zijnde het ongerief en de schending van integriteit en de beëindiging van het leven van de vermelde proefdieren. Dit hebben we ook naar voor gebracht in ons PV onder kopje 3.3.1.

3.1 Achtergrond | Background

2. U wilt maar liefst 4 modellen van darmpathologie onafhankelijk van elkaar aanwenden zowel in bijlage 2 als 3. Is het mogelijk deze 4 modellen van darmpathologie afhankelijk van elkaar te maken en wel zodanig dat dit resulteert in go/no go criteria in deze afhankelijkheid? Met andere woorden zouden de resultaten verkregen uit een bepaald model van darmpathologie ertoe kunnen leiden dat toepassing van een ander model van darmpathologie niet zinvol meer is?

In appendix 2 wordt onderzoek gedaan naar colorectaal kanker. In onze appendix beschrijven wij 4 verschillende modellen voor het nabootsen van deze pathologie. Het is echter niet de bedoeling dat al deze modellen gebruikt worden voor elk van de beschreven outcome parameters. Zoals beschreven in appendix 2 op pagina 3-4, zijn de AOM en AOM/DSS modellen onze voorkeur modellen, omdat we door het gebruik van deze chemische stoffen succesvol tumoren in de colon kunnen laten ontwikkelen (Figuur 2, PV). Het enige verschil hierbij is dat t.o.v. het AOM model, het AOM/DSS model gebaseerd is op de ontsteking gedreven ontwikkeling van darmkanker. Dankzij het APC model hebben we ook een model waarbij de proefdieren, als gevolg van een genetisch defect in het APC gen, tumoren ontwikkelen in de dunne darm. Het orthotope model, het enige model waarbij we ook de metastasering van de darmkanker cellen kunnen bestuderen is het enige valide model om te gebruiken voor de experimenten met chemo- of radiotherapie, hetgeen werd aangegeven in figuur 4 (PV) als go/no go moment. In conclusie, in appendix 2 zijn deze modellen niet afhankelijk van elkaar, en zullen ook niet allemaal voor eenzelfde experiment gebruikt worden. We verwijzen hiervoor naar figuur 2 in appendix 2 met de specifieke go/no-go criteria, die we nog verder verduidelijkt hebben.
In appendix 3 is er wel enige afhankelijk van de modellen, waarvoor we opnieuw specifieke go/no-go criteria gedefinieerd hebben voor het gebruik van de verschillende gastro-intestinale ziekte modellen (PV, figuur 5). In deze appendix zal op basis van de resultaten van het H. Poly (worminfectie) model worden bepaald welke vervolgmodellen zinvol zijn om verder te onderzoeken. Bijvoorbeeld, indien we statisch significante verschillen

ondervinden in de samenstelling van het microbioom, zullen we verder gaan met het model waarin we het microbioom zullen veranderen. Dus, de resultaten verkregen uit het H. Poly model kunnen ertoe leiden dat andere modellen niet meer worden toegepast. Dit is verder omschreven in de Strategy sectie van de PV en appendix 3, pg 3-4.

3. Wat is al bekend, of wat zou bekend kunnen worden, uit onderzoek in humane patiënten m.b.t. up- en downregulatie van specifieke genen (mogelijke biomarkers)? In hoeverre werkt u samen met partners met kennis op dat gebied?

In 2009 heeft onze groep ontdekt dat NDRG4f een accurate diagnostische biomarker is voor dikke darmkanker (5.1 lid2e, 5.1 lid2h et al., JCI 2009). Vervolgens hebben wij aangetoond dat NDRG4 specifiek tot expressie komt in het ENS (5.1 lid2e, 5.1 lid2h et al., NGM 2017). Wij werken samen met verschillende partners binnen het 5.1 lid2h Hierdoor hebben wij toegang tot humaan CRC tumor materiaal en kunnen wij hieruit ENS cellen isoleren om o.a. gen expressie te meten en te sequeneren in de CRC context. Verder hebben wij toegang tot coupes van grote CRC patiënten series die we gebruiken om onderzoek te doen naar het potentieel van ENS specifieke genen als prognostische biomarkers. Voor gelijkaardige studies werken we ook samen met de afdeling maagleverdarm ziekten (gastro-enterologie - 5.1 lid2h Prof. 5.1 lid2e, 5.1 lid2h en de groep van Prof. 5.1 lid2e, 5.1 lid2h Hierdoor hebben we toegang tot humane stalen (endoscopische biopten van maag en darmen) van gezonde vrijwilligers en patiënten met functionele en inflammatoire darmziekten. Echter willen we hier benadrukken dat de ontdekking van genen met een veranderde expressie, die dus de mogelijkheid bieden om als biomarker te dienen, niet per se een effect hebben op het ontstaan of het verloop van de ziekte. Met dit onderzoek willen wij daarom ook niet alleen begrijpen hoe het ENS normaal functioneert en is georganiseerd, maar beogen we ook om de ontstaansmechanismen van darmziektes beter te begrijpen. We zullen ons hiervoor dus niet beperken tot enkel tot het opsporen of detecteren van mogelijke up- of downregulatie van bepaalde genen. We zullen veel dieper duiken.

4. Het is voor de 5.1 lid2h duidelijk waarom de onderzoekers willen nagaan hoe ENS reageert in de context van fysiologische veranderingen (gezond en ziekte), echter de link tussen ENS homeostase en andere delen van het zenuwstelsel is onvoldoende belicht in de achtergrond (zeker in context met de doelstelling van dit projectvoorstel).

Omdat meermaals bleek dat onze beschrijving van mogelijke linken met andere delen van het zenuwstelsel te kort schoot, hebben we besloten om de experimenten die hiertoe dienen te schrappen uit het huidige project.

5. Kan de onderzoeker verder specifiëren wat hij bedoelt met "holistic and advanced approach"?

Het ENS werkt samen en communiceert met veel andere systemen (bijv. immuunsysteem, microbioom, etc.). Om zoveel mogelijk van deze systemen mee te nemen en hun interacties met het ENS verder te onderzoeken gebruiken wij muismodellen en technieken die ons toelaten om dit in zijn geheel te bestuderen, zodat het zo goed mogelijk de daadwerkelijke pathologische situatie nabootst.

6. De 5.1 lid2h vraagt om aan te geven welke resultaten behaald zijn uit het vorige PV – en deze ook te beschrijven, beschreven in doelstelling 2, zodat duidelijk wordt waarom en welk verder onderzoek noodzakelijk is.

Om hieraan te voldoen hebben we figuur 2 vanuit appendix 2 verplaatst naar het PV, zodat we op pagina 2-4 de resultaten uit de vorige PV tonen samen met figuur 1 (zoals ook aangegeven in de redactionele opmerkingen). Verder is aan het eind van de background sectie een stukje toegevoegd om deze vraag te beantwoorden (PV, pagina 5).
7. Wat is de reden dan u zo veel verschillende modellen wilt gebruiken?

Verschillende darmziektes kunnen een ander effect hebben op het ENS (op de werking en/of de organisatie). Hierdoor kan het ENS een andere rol spelen afhankelijk van welk ziektebeeld er zal optreden. Omdat we binnen onze groep niet alleen op darmkanker focussen, maar ook gerichter willen kijken naar andere gastro-intestinale ziektes zoals (darmontstekingen), hebben wij een heel breed projectvoorstel, met verschillende diermodellen, beschreven om ervoor te zorgen dat de projecten van verschillende onderzoekers binnen onze groep gefaciliteerd kunnen worden. In het bovengenoemde antwoord op vraag 2, hebben we reeds meer uitleg beschreven over de keuze en samenhang van de verschillende modellen binnen een appendix.

8. Is er tussen de 3 subdoelen nog onderlinge afhankelijkheid in de vorm van bv. go- no go's?

Wij zijn van mening dat er geen afhankelijkheid tussen de 3 subdoelen bestaat. Hoewel de in vitro studies uit subdoel 1 reeds meer inzicht kunnen geven in de onderliggende mechanismen/targets waaraan we in vivo extra aandacht moeten geven, is het hier niet mogelijk om specifieke go/no go's te definiëren. We gaan ervan uit dat de resultaten verkregen uit de in vitro *en* in vivo *studies elkaar op een belangrijke manier zullen complementeren. Met subdoel 2 en 3 onderzoeken we de functie en organisatie van het ENS in compleet verschillende ziektebeelden. Deze subdoelen zijn daarom dus onafhankelijk van elkaar.*

3.2 Doel | Purpose

9. De (prima) tekst in 3.1 Background vertelt eigenlijk ook meteen dat uw 'ultimate goal' bijlange na niet bereikt kan worden in dit project vanwege de enorme complexiteit. Is het wel realisitsch om een ultimate goal te benoemen? De laatste zin uit deze paragraaf is realistisch en wellicht voldoende?

We gaan akkoord met deze suggestie en hebben de beschrijving van het ultieme doel aangepast.

10. In 3.2.2 rechtvaardigt u de haalbaarheid van het project (ook) door te zeggen dat het mogelijk niet beperkt blijft tot het beschrevene, maar in samenwerking met andere groepen ook breder getrokken kan worden. Is dat geen wonderlijk argument?

Hoewel het ENS de spilfiguur is in onze onderzoeksprojecten, laten de modellen die we gebruiken ook toe om andere systemen (zoals immuunsysteem, microbioom, CNS, andere celtypes in de darm etc.) te onderzoeken. Dit geeft daardoor extra nut aan de proefdieren en kan ons onderzoek versterken door verschillende systemen met elkaar te integreren in de verschillende pathologische condities (zoals hierboven beschreven bij vraag 3, hebben we al verschillende samenwerkingen lopen). Dit hebben we nu proberen te verduidelijken onder punt 3.2.2.

11. Vanuit de doelstelling is het niet duidelijk waarom in aim 1 'nieuwe' proefdieren dienen gebruikt te worden, en dit niet kan met dieren die al beschikbaar zijn vanuit andere experimenten of met commercieel te verkrijgen cellen? Wat is de reden dat cellen enkel geïsoleerd worden van een non-diseased gut?

Dit staat inderdaad niet duidelijk beschreven in de doelstelling maar hebben wij beschreven in appendix 1. Om dit te verduidelijken hebben we bij Aim 1 nu verwezen naar appendix Om voldoende ENS cellen te verkrijgen voor een kweek zijn hele darmen nodig en daarom kan dit vaak niet gecombineerd worden met andere experimenten. We zullen ook commercieel verkrijgbare cellijnen gebruiken, maar dit zijn cellijnen en deze zijn daarom niet representatief voor de fysiologische condities. Cellen uit 'diseased' guts zijn geïncludeerd in appendix 2 en 3, omdat dat de appendices zijn waar de ziektemodellen in staan beschreven. In appendix 1 worden alleen 'non-diseased' guts gebruikt, maar deze

kunnen wel in vitro in ziekteomgevingen gebracht worden door het gebruik van bijvoorbeeld geconditioneerd medium of co-culturen met andere cellen.

12. Welke redenen zijn er om aan te nemen dat in aim 3 een worm-, microbiota- én inflammatie setting nodig is?

De worm-setting wordt gebruikt als eerste model en gebaseerd op de resultaten van dit model zal worden bepaald (go/no go moment) of en welke modellen verder gebruikt gaan worden. Zie ook figuur 5 in de Strategie waarin dit go/no go moment wordt uitgelegd. Zoals hierboven beschreven (Vraag 2) zullen de resultaten van dit model bepalen of er al dan niet wordt verder gegaan met de microbiota en inflammatie modellen.

13. Vanuit de sub-aims is het niet duidelijk dat er ook gekeken wordt naar de link tussen ENS en andere delen van het zenuwstelsel. Kunt u deze link ook hier duidelijker beschrijven?

Omdat meermaals bleek dat onze beschrijving van mogelijke linken met andere delen van het zenuwstelsel te kort schoot, hebben we besloten om de experimenten die hiertoe dienen te schrappen uit het huidige project.

3.3. Belang | Relevance

14. Is ENS en CNS vergelijkbaar qua samenstelling, organisatie en functie? Vanuit de achtergrond lijkt dit niet zo te zijn, wat de vertaalbaarheid tussen beide systemen in vraag stelt.

Deze systemen zijn inderdaad niet 1 op 1 vergelijkbaar, maar vergeleken met andere systemen in het lichaam lijken ze wel erg veel op elkaar qua complexiteit en constituerende celtypes (Gershon MD., Hosp Pract 1995). Wat we hier bedoelen is daarom niet dat onze resultaten direct vertaald kunnen worden naar het CNS, maar dat het wel ideeën kan geven over mogelijke processen/interacties die ook in het CNS een rol zouden kunnen spelen.

3.4 Strategie | Strategy

3.4.1 Overzicht algemene projectopzet | Overall design project strategy

15. Het wordt uit de strategie-bechrijvingen onvoldoende duidelijk hoe de studies uit App.1 van nut zijn voor de studies in App.2 en App3. Kan dat verband verduidelijkt worden?

In vitro kunnen we gemakkelijker veranderingen (bijvoorbeeld het toevoegen van bepaalde stoffen of het activeren/inhiberen van genen) induceren en het effect van deze veranderingen onderzoeken. Hiermee kunnen we een idee krijgen van mogelijke effecten maar ook mechanismen in detail onderzoeken. Dit kan ons daarom helpen om aim 2 en 3 te bereiken en complementeert daarmee de in vivo studies. Het is echter belangrijk om te noemen dat een in vitro setting een simplistische weergave is, die niet even representatief is als de in vivo setting. Daarom hebben we beide systemen nodig. In de tekst van de Strategy sectie is dit verband ook verduidelijkt (PV, pagina 7-8).

16. In de tekst over App3. staat o.a. het kopje 'Brain/CNS phenotype'. Het is niet duidelijk uit de flow charts hoe jullie daarop uit zouden kunnen komen. Wanneer zullen gedragsstudies en (hersen?) imaging aan de orde komen? (en is er voldoende ervaring voor aanwezig in uw lab met de voorgestelde testen: object novel object recognition, fear conditioning, object location memory task, maze spontaneous alternation test)? Waar is uw keuze voor deze testen op gebaseerd? (in de bijlage: hoe gaat u de test uitvoeren?)
Omdat meermaals bleek dat onze beschrijving van mogelijke linken met andere delen van het zenuwstelsel te kort schoot, hebben we besloten om de experimenten die hiertoe dienen te schrappen uit het huidige project.

17. Wat is de reden dat u de klassieke tumorbehandelingen (radiation, chemo) wilt gaan inzetten bij de behandeling van CRC in uw modellen? Zou het voor u niet interessanter zijn om u te richten op een of andere beïnvloeding van het ENS? Áls u het gaat uitvoeren: zou het dan nuttig zijn een controlegroep mee te nemen van dieren zonder CRC?

In deze PV willen we niet alleen onderzoeken of een veranderd ENS tumorontwikkeling zelf beïnvloedt, maar ook of dit de efficiëntie van tumorbehandeling beïnvloedt. We gaan bijvoorbeeld dieren met minder enterische neuronen vergelijken met dieren met een normaal aantal enterische neuronen, waardoor we kunnen onderzoeken of veranderingen in het ENS bijdragen aan een betere tumorbehandeling met radio- of chemotherapie. Hoewel het interessant is om het ENS te beïnvloeden is dit op dit moment nog een veel te voorbarige methode omdat er geen enkele duidelijke aanwijzing is om een specifiek ENS-molecuul of specifiek cel type te targetten dat een voldoende therapeutisch effect kan hebben tijdens darmziektes. Daarnaast is er voorlopig ook nog geen enkele efficiënte methode die toelaat om deze targets enkel in het ENS te beïnvloeden.

18. In het strategie-deel voor App.3 staat ook als optie genoemd '(epi)genetic editing (AAVxcarrying genetic modifiers)'. Kunt u verduidelijken hoe hier keuzes in gemaakt gaan worden ? (wanneer nietl, wanneer wel, welke dan?)

Dit model zal gebruikt worden als er in het worminfectie model, specifiek in de ENS cellen een verandering in genexpressie optreedt en de functie van deze genen aanleiding geeft om met betrekking tot het ENS en darmfunctie verder te onderzoeken door middel van genetic editing. We zijn hier specifiek geïnteresseerd in miRNA's, omdat deze een simultaan effect kunnen hebben op meerdere genen en daardoor een prominente rol kunnen spelen in pathologische condities.

19. Bij het beschrijven van de sub-aims binnen de strategie, komt CNS nergens meer ter sprake. Wordt CNS wel of niet onderzocht binnen huidige aanvraag?

Omdat meermaals bleek dat onze beschrijving van mogelijke linken met andere delen van het zenuwstelsel te kort schoot, hebben we besloten om de experimenten die hiertoe dienen te schrappen uit het huidige project.

20. Voor aim 2 en 3 (figuur 2) worden verschillende manieren beschreven om CRC of inflammatie te induceren. Het is de 5.1 lid2h echter niet duidelijk waarom deze manieren en welke combinaties hiervan gekozen worden. Nu lijkt alles te kunnen…
In figuur 2 hebben we geprobeerd een algemeen overzicht te geven met de procedures die de dieren in deze appendix kunnen ondergaan. Deze procedures worden niet allemaal op hetzelfde dier uitgevoerd, maar zullen wel allemaal gebruikt worden binnen deze appendix. In de desbetreffende appendix staat daarna beschreven welk procedures specifiek worden gebruikt voor elk model en waarom deze modellen worden gebruikt (zie section proposed animal procedures, appendix 2 and 3).

21. Op welke basis zijn max 10 cel-cel interacties en 7 pathways geselecteerd voor aim 1? Refereren naar p2-4 lijkt dit onvoldoende te onderbouwen. Zelfde vraag voor waarom 4 CRC modellen en 4 inflammatie modellen (max 4 model 1, max 2 genotypes model 2).

Gebaseerd op het feit dat het ENS interageert met verschillende celtypen, zoals epitheel, endotheel, spier en immuuncellen, om de darmhomeostase te behouden (Furness et al., Nat Rev Gastroenterol Hepatol 2012), willen wij deze verschillende cel-cel interacties, bestuderen. Op basis van onze studies met NDRG4 zijn een aantal pathways naar boven gekomen die we in die context graag verder willen onderzoeken (bijvoorbeeld snap25, pAKT, ERK). Omdat deze verschillend kunnen zijn in de verschillende ziektebeelden en modellen, hebben wij afgaande op onze eerdere studies en interacties bekent uit de literatuur een schatting gemaakt van het maximaal aantal cel-cel interacties en pathways dat we willen bestuderen.Zoals beschreven bij vraag 2 hierboven zijn de CRC modellen

verschillend op vlak van locatie van tumor ontwikkeling (dunne darm tov dikke darm) en de manier waarop deze tumoren geinduceerd worden (chemisch vs genetisch). Daarom zullen deze gebruikt worden om elkaar aan te vullen. Ook hangt de keuze voor het model soms af van het experiment, omdat niet alle modellen gebruikt kunnen worden op dezelfde manier. Dit is verder beschreven in appendix 2 (pagina 3-4). Voor appendix 3 kunnen er verschillende modellen gebruikt worden, maar dit hangt af van het beschreven go/no-go moment (figuur 5, pagina 12 PV). Verder zullen in deze appendix verschillende muismodellen gebruikt worden binnen deze ziektemodellen. Omdat we dankzij de verkregen resultaten uit het wormmodel specifieker kunnen selecteren welke modellen/genen belangrijk zijn, zullen we hier na go/no-go minder genotypes moeten gebruiken.

22. Figuur 3 lijkt te suggereren dat orthotoop CRC model enkel gebruikt wordt voor therapie doeleinden. Waarom niet voor andere uitleesparameters? Kan AOM, AOM/DSS ofwel APC model hiervoor niet gebruikt worden of leiden deze modellen niet tot onvoldoende adenocarcinoma vorming? Waarom orthotoop CRC model niet gebruiken voor alle onderzoeksvragen?

We hebben in het lab veel ervaring met de AOM, AOM/DSS en APC modellen en gebruiken deze al langere tijd (zie resultaten beschreven in figuur 1 en 2 in het PV). Om te onderzoeken wat het effect van het ENS is op darmkanker ontwikkeling en vice versa willen we geen gebruik maken van een orthotoop model, omdat deze tumoren dan niet in het milieu met een veranderd of gelabeld ENS tot stand zijn gekomen. Met een orthotoop model kan je deze interactie dus niet voldoende bestuderen. Voor therapie zullen we het orthotope model mogelijk wel nodig hebben, omdat we de tumor dan beter kunnen placeren en volgen. Dit is niet mogelijk met tumoren die chemisch of genetisch geïnduceerd zijn. Ook is dit het enige model waarin we metastasen van de tumoren zullen kunnen observeren.

23. Welke chemo/RT combinatie voorziet met voor CRC? Deze combi is klinisch gezien enkel relevant bij adenocarcinomas in vergevorderd stadium.

Aangezien geen enkel van onze modellen aanleiding geeft tot de ontwikkeling van vergevorderde adenocarcinomen, heeft het geen nut om een combinatie therapie toe te passen. We zullen dus geen combinatie van radiotherapie en chemotherapie gebruiken. Dit werd niet meegerekend in de hoeveelheid dieren, maar was nog niet overal in de tekst verwijderd. We hebben dit nu wel overal verwijderd in de tekst.

24. Figuur 4 lijkt enkel van toepassing te zijn voor worminfecties. Zijn andere manieren om inflammatie te induceren niet meer relevant hier?
Worminfectie is het belangrijkste model in appendix 3. We zullen dit model als eerste toepassen, omdat wij en onze collega's veel ervaring hebben met dit model en omdat dit model een effect heeft op gastro-intestinale homeostase (breder dan andere inflammatie modellen) en het ENS. Op basis van deze resultaten bekomen met dit model (go/no-go) wordt bepaald of er andere inflammatie modellen (zie figuur 5, linksonder) relevant zijn voor het beantwoorden van de onderzoeksvragen voor deze appendix. Als we een specifiek effect zien met betrekking tot inflammatie in dit model, zullen andere manieren van inflammatie induceren gebruikt worden om dit specifiek verder te onderzoeken.

Appendix 1 Description animal procedures
A. Experimentele aanpak en primaire uitkomstparameters | Experimental approach and primary outcome parameters

25. De 5.1 lid2h mist een doeltreffende beschrijving van de outcome parameters (bv.: het maken van 3D cultures is de methode, geen outcome parameter). Graag een doeltreffende beschrijving van de outcome parameters geven.

De beschrijving voor de outcome parameters is veranderd in de tekst van appendix 1 (pagina 1).

26. U wilt postnatale dieren gebruiken om cellen van te verkrijgen. Middels welke procedure gaat u deze cellen verkrijgen?

Dieren zullen worden gedood en het colon zal worden verwijderd, waarna vervolgens door middel van dissectie en specifieke dissociatiemethodes en cultuur media de juiste cellen in kweek zullen worden gebracht (Schonkeren et al., 2022).

27. "Potential examples": indien er in de toekomst betere modellen verschijnen, welke waarde hebben de behaalde resultaten dan in context van aim 2 en 3? Is het niet zo dat eens een model gekozen is voor aim 1, het raadzaam is om hetzelfde model te gebruiken in aim 2 en 3? Dit raakt ook aan de onderlinge samenhang tussen de bijlagen/subdoelen.

Dat ligt aan de mate waarin dit model al is gebruikt voor de verschillende onderzoeken en welke resultaten hiermee zijn verkregen. Als een specifiek resultaat is gevonden in vitro (appendix 1) en dit moet verder onderzocht worden in vivo (appendix 2 en 3), dan is het raadzaam om hetzelfde model te gebruiken. Omgekeerd geldt hetzelfde. Als echter nog niet met een bepaald model begonnen is en er is een beter model beschikbaar dan zullen we dat model vanaf het begin gaan gebruiken. Verder kunnen er erg vergelijkbare modellen komen die efficiënter zijn (bijv. in het labelen van bepaalde cellen), dan kan dit nog steeds tijdens de experimenten veranderd worden zonder nefaste beïnvloeding van de resultaten.

28. U beschrijft geen statistische methode om het aantal dieren te berekenen.

Het aantal dieren is gebaseerd op ervaring en de hoeveelheid cellen/dieren dat nodig is voor onze kweekprotocollen uit ons vorige PV. Er is dus geen specifieke statistische formule gebruikt om het aantal dieren te berekenen, omdat het aantal dieren is gebaseerd op de duur van deze PV en het aantal dieren dat nodig is voor elke kweek om deze lopende te houden gedurende deze periode.

29. Is het haalbaar om elke week een culturing procedure uit voeren en deze ENS cellen 10 dagen in kweek te houden met alle bijhorende read-outs?

We achten dit haalbaar aangezien deze PV overkoepelend is voor 4 projecten, waardoor er dus meerdere mensen tegelijkertijd aan zullen werken. Verder zullen er soms meerdere culturen tegelijk moeten lopen om genoeg cellen te hebben voor bepaalde experimenten.

30. Waarom voor neurospheres enkel 2 genotypes, terwijl voor ENS 11 genotypes en organoids 6 genotypes?

Neurospheres dienen een ander doel dan de ENS en organoid cultures. In de ENS en organoid cultures willen we onderzoek kunnen doen in dezelfde modellen die we ook voor de in vivo studies gebruiken. Deze cellen kunnen gelabeld of veranderd zijn afhankelijk van het model. Bij de jonge muizen die voor neurospheres gebruikt worden is dit vaak nog niet het geval en is het vooral belangrijk dat we deze ENS (progenitor) cellen kunnen isoleren om neurospheres te vormen en hier onderzoek mee te doen.

B. De dieren | The animals
31. De aantallen in de tabel en in de tekst lijken niet met elkaar overeen te komen.

Het totaal aantal is 1322; 500 + 550 voor ENS cultures (bovenste rij voor elk diermodel in de tabel, dus 500 + 100 + 50 + 300 + 100), 200 voor neurospheres (2e rij voor bovenste diermodel in de tabel), en 72 voor organoids (2e rij voor 3e diermodel in de tabel).

D. Pijn en welzijnsaantasting | Pain and compromised animal welfare
32. Er wordt aangegeven dat er mogelijks andere nevenwerkingen zijn die het welzijn van de dieren aantast, graag aangeven waarom deze kunnen voorkomen (ipv NA).

Dit is nu in de appendix (pagina 6) toegevoegd.

E. Humane Eindpunten | Humane endpoints
33. Zijn er mogelijks HEPs te verwachten bij de modellen waarbij geen "adverse phenotype" is beschreven? Zijn er HEPs te verwachten bij verkeerde toediening substances en labeling agents?

Uit ervaring en vanuit de literatuur, verwachten we geen specifieke HEPs bij de gebruikte modellen waar geen adverse phenotype beschreven is. Daarnaast hebben we de genoemde stoffen al vaak toegediend (vb in pv2017-026) zonder consequenties of het bereiken van HEPs. We gaan er daarom vanuit dat we niet meer kans hebben om HEPS te moeten gebruiken dan door toeval wordt veroorzaakt.

G. Vervanging, vermindering, verfijning | Replacement, reduction, refinement
34. Er wordt aangegeven dat dieren kunnen gebruikt worden vanuit fok specifiek voor aim 2 en 3. Worden dieren dan dubbel geteld?

Dit is niet het geval. Als dieren uit de fok voor een experiment in appendix 2 en 3 niet kunnen worden gebruikt voor die experimenten (bijvoorbeeld door het genotype of doordat er te veel muizen zijn gefokt), dan kunnen ze nog voor experimenten in appendix 1 gebruikt worden en zijn ze niet gefokt zonder gebruikt te worden. Dit zou de hoeveelheid dieren die gefokt wordt maar niet gebruikt kunnen verminderen, maar dieren worden hier dus niet dubbel geteld.

Appendix 2 Description animal procedures
Algemeen
35. U laat zien dat: "the mouse lines and disease models are appropriate for studying the same outcome measures in other mouse lines and new outcome measures on both already in use and new mouse lines". Heeft u deze uitkomsten ook geverifieerd (in de mate waarin dat mogelijk is) in mensen met/zonder colorectaalkanker? In hoeverre komen de uitkomsten bij muizen en mensen overeen? Met andere woorden: hoe vertalen deze resultaten zich naar de mens?

Waar mogelijk, maken wij ook gebruik van humaan materiaal, maar de beschikbaarheid is gelimiteerd en het is lastig om het ENS te bestuderen in mensen. Hiervoor zijn de muismodellen noodzakelijk en ook veel gebruikt in dit onderzoeksveld. We zijn er daarom van overtuigd dat dit genoeg potentieel heeft om zich te vertalen naar de humane situatie. Verder zullen we zoveel mogelijk humaan materiaal (biopsies, weefsels en cellen) gebruiken om onze resultaten te verifiëren in de humane setting.

A. Experimentele aanpak en primaire uitkomstparameters | Experimental approach and primary outcome parameters

36. Wat is de onderbouwing voor deze uitleesparameters?

De uitleesparameters volgen uit de background informatie van het PV en zijn daar geïntroduceerd. We hebben in appendix 2 (pagina 5) een korte algemene onderbouwing toegevoegd. In de daaropvolgende pagina's worden de uitleesparameters in meer detail beschreven.

37. In de eerste paragraaf komt CNS even terug, voor de rest afwezig in deze appendix.

Omdat meermaals bleek dat onze beschrijving van mogelijke linken met andere delen van het zenuwstelsel te kort schoot, hebben we besloten om de experimenten die hiertoe dienen te schrappen uit het huidige project.

38. Waarom worden dieren aangevraagd voor experimenten die ook omschreven zijn binnen vorig PV, welke nog loopt? Worden de experimenten beschreven in vorig PV niet meer uitgevoerd, hoewel er nog een jaar looptijd is?

We hebben hier enkel dezelfde diermodellen aangevraagd als in ons vorige PV als er nieuwe outcome parameters zijn toegevoegd die niet in onze huidige PV staan of we hebben voor dezelfde uitcome parameters nieuwe diermodellen aangevraagd. Een uitzondering hierop zijn de dieren voor therapie. Deze experimenten zijn niet meer haalbaar in de lopende PV en zijn dus verplaatst naar deze aanvraag.

39. Wat is de noodzaak van de controlegroep (geen kanker inductie), aangezien de vraagstelling gaat over ENS+/- tov WT dieren?

Deze stelling is niet correct voor alle muismodellen die we hebben beschreven. Voor de Sox10-CreERT2;tdT muizen bijvoorbeeld willen we juist gezonde colon met tumor colon vergelijken en daarin zien hoe de enterische glia cellen (die rood gelabeld zijn) zich gedragen in deze milieus. Hiervoor zijn dus ook muizen zonder kankerinductie nodig. Voor bijvoorbeeld NSE en Hand2 deficiënte dieren is dit inderdaad wel correct en zijn dus ook geen groepen zonder kankerinductie opgenomen.

40. Welk model leidt tot welk stadium tumoren (poliep, adenoma, adenocarcinoma)? Dit in context van toekomstige chemo/RT welke enkel klinisch relevant is voor advanced CRC.

De modellen leiden tot poliepen en adenomen (laaggradig en hooggradig) en sporadisch een adenocarcinoom. In de context van chemo/radiotherapie zullen we echter ook gebruik maken van orthotope modellen, waarin we de klinisch relevante situatie beter kunnen nabootsen.

41. AOM en AOM/DSS heeft aanleiding tot maximaal 10 poliepen (figuur 1), echter bij CRC modellen wordt tot 40 poliepen aangegeven?

Het aantal poliepen varieert per CRC model en ook per muismodel, omdat de muizenstam invloed kan hebben op de vatbaarheid voor de kankerinductie met AOM en DSS. Verder ligt het eraan hoelang de muizen in het protocol blijven, de data in figuur 2 laat nog niet het aantal poliepen zien op het moment van het doden van de dieren, maar gedurende het experiment (de desbetreffende figuur is verplaatst naar het PV, pagina 4, zoals voorgesteld bij de redactionele opmerkingen).

42. Het is onduidelijk welk model gebruikt zal worden voor welke vraagstelling. Kan dit verduidelijkt worden?

We hebben geprobeerd dit verder te verduidelijken in de tekst onder de beschrijving van de verschillende modellen (appendix 2, pagina 4).

43. Wat wordt bedoeld met "Frequency: max to be determined (range 5-10)": tumoren die bestraald worden (wat gebeurt er met de rest?), aantal bestralingen per dier, aantal CTs? Welke dosis denkt u te gebruiken (aangezien darm een stralingsgevoelig orgaan is, met grote kans op darm mucosal toxiciteit en crypt depletie)

Dit zal allemaal bepaald worden in pilootexperimenten, die opgenomen zijn en beschreven staan in onze lopende PV.

44. Aangezien er verschillende aantallen beschreven worden, wil zeggen dat s en d bekend zijn en statistische onderbouwing mogelijk is middels powerberekening. Kunt u die toevoegen?

Deze aantallen zijn gebaseerd op literatuur en onze ervaring met de verschillende modellen en outcome parameters vanuit onze vorige PV.

45. Waarom komen WT groepen meermaals voor binnen de verschillende experimenten?

Omdat ze als controlegroep gebruikt worden voor verschillende experimentele groep: we hebben hiervoor WT dieren nodig die tegelijk in dezelfde omstandigheden in hetzelfde experiment zitten.

46. Klopt het dat er geen dieren worden aangevraagd voor de combinatie chemo + RT?

Ja dat klopt, we willen alleen het effect van radio- of chemotherapie apart onderzoeken. Hoewel dit niet meegerekend was in het aantal dieren stond het wel nog op een aantal plaatsen in de tekst. Dit werd nu verwijderd.

47. 10 CTs leidt tot een cumulatieve bestralingsdosis (whole body) van minimaal 3 Gy. Welk effect heeft dit op de uitleesparameters van de desbetreffende experimenten? En op het ongerief? Wat is de stralingsgevoeligheid van de muizenstammen die u wilt gebruiken?

Ongerief zal zoals beschreven, moderate-to-severe zijn. De pilootexperimenten die onder de huidige PV beschreven staan, zullen echter gebruikt worden om het effect van de therapie te bestuderen en het ongerief zoveel mogelijk te beperken. Om de stralingsgevoeligheid, de dosis en frequentie te bepalen zullen we samenwerken met andere onderzoekers die vergelijkbare experimenten eerder hebben uitgevoerd.

48. Wat is de reden dat u zoveel verschillende diermodellen voor eenzelfde type kanker wilt gebruiken?

Deze appendix bevat diermodellen voor 3 verschillende projecten die allemaal op hun eigen manier de interplay tussen het ENS en CRC bestuderen. Ze bestuderen echter allemaal een ander deel van het ENS netwerk en de interactie met CRC.

49. U geeft aan bij de APCMin/+ muizen na 6 maanden ca 30 tumoren te vinden in de darmen, wat u inschat als mild ongerief. De 5.1 lid2h vraagt zich af of dit terecht is? Hoe groot zijn de tumoren ongeveer? Is er gevaar voor darmafsluiting? Wanneer gaat u de dieren doden?

De poliepen in dit model zijn klein en uit ervaring weten wij dat de muizen daar gewoonlijk weinig last van hebben. We doden de dieren voordat erger ongerief zal worden ervaren. Darmafsluiting zal hierdoor normaliter niet voorkomen en een prolaps zal ook niet optreden omdat de poliepen niet in de colon zitten. Wij hebben dit ook nog nooit geobserveerd bij experimenten uitgevoerd onder onze huidige PV.

50. Hoelang blijven de dieren na de inductie van tumoren in leven?

Dat varieert per kankermodel, aangezien de tumor ontwikkeling niet even snel gaat in alle modellen. Daarnaast kan dit ook verschillen per muizenstam. We hebben daarom protocollen van verschillende lengtes voor de verschillende kankermodellen en muisstammen toegevoegd. Deze worden op basis van ervaring met onze huidige PV hier verder toegepast.

51. Hoelang blijven de colitis-symptomen bij de inductie van poliepen door chemische inflammatie?

De acute colitis-symptomen zijn van korte duur (ongeveer 1 week na elke cyclus). Op het moment dat we de muizen doden en naar het weefsel kijken zijn er vaak enkel nog tekenen van weefselherstel na inflammatie zichtbaar.

52. Kan ernstig ongerief worden vermeden in het orthotope colonkankermodel?

Het ernstige ongerief in dit model zal vooral veroorzaakt worden door de therapieën en procedures die de dieren moeten ondergaan. Zoals beschreven onder vraag 47, gaan we met onze huidige PV pilootexperimenten uitvoeren om deze technieken te optimaliseren en het ongerief zo laag mogelijk te houden, zodat we dus inderdaad proberen ernstig ongerief te vermijden.

53. U geeft aan dat bestraling en behandeling met chemotherapie van de muizen tot "moderate (to severe)' ongerief kan leiden. Verwacht u hier maximaal ernstig of maximaal matig ongerief?

We hebben hier op dit moment nog maximaal ernstig ongerief beschreven. We gaan echter met de pilootstudies onder onze huidige PV deze procedures optimaliseren en daarmee verwachten we, maar zijn we niet geheel zeker dat, het ongerief terug te kunnen brengen naar matig ongerief.

54. Als u alleen bestraling of chemotherapie al op matig-ernstig ongerief inschat, zou een combinatie van beiden dan niet leiden tot ernstig ongerief?

We gaan geen combinatie therapie gebruiken binnen deze PV, omdat de tumoren niet in zo'n vergevorderd stadium zullen zijn. Hiervoor waren ook geen muizen geïncludeerd, maar de nog foutieve aanwijzingen hiernaar zijn nu ook verwijderd in de tekst.

B. De dieren | The animals

55. Het is niet duidelijk waarom alle experimenten dienen te gebeuren in alle CRC modellen.

Dit is in eerste instantie ook niet de bedoeling. Zoals nu verduidelijkt in de tekst onder experimentele procedure en in het antwoord op vraag 42, zullen we de modellen kiezen op basis van het experiment. AOM en AOM/DSS zijn hierbij onze voorkeursmodellen (ook beschreven onder vraag 2). We zullen echter overgaan op het APC model als we een beter inzicht willen krijgen in genetische tumor ontwikkeling die ook vergelijkbaar is met de humane situatie (APC mutatie). Het orthotope model zal met name gebruikt worden voor de therapie studies.

56. Welke piloot studies op het huidig lopende PV zullen nog gebeuren en dienen hierbij dan niet aangevraagd te worden?

Piloot studies om de kankermodellen te testen en te optimaliseren, alsook de piloot studies voor de experimenten waarbij therapie gebruikt zal worden, zijn of zullen, op de lopende PV uitgevoerd worden. Hiervoor zijn dus ook geen dieren aangevraagd op deze nieuwe PV.

D. Pijn en welzijnsaantasting | Pain and comprimised animal welfare

57. U schrijft 'All possibilities to reduce pain, fear or suffering will be used. These include use of appropriate analgesia and anaesthesia that do not affect gut motility, lead to gut abnormalities or affect the intestinal inflammatory response.' Kunt u concreet maken wat er dan resteert aan opties? En kunt u aangeven wanneer in het opwekken van CRC dit relevant is/wordt?

We verwachten geen langdurige pijn die het gebruik van analgesie noodzakelijk maakt binnen deze modellen en we voeren hier ook geen procedures uit waarbij pijnstilling gebruikt moet worden. We hebben hier ook tijdens de experimenten onder onze huidige PV nog geen gebruik van gemaakt. Mocht er toch aanleiding zijn om pijnstilling te gebruiken, dan zullen we carprofen gebruiken waarvan bekend is dat de bijwerkingen op de darm minimaal zijn. Voor de CT scans gebruiken we isofluraan als anestheticum. Deze informatie is ook toegevoegd aan appendix 2 (pagina 10).

E. Humane Eindpunten | Humane endpoints

58. RT en chemo kunnen leiden tot langdurige diarree door het effect op gezond weefsel. Geldt dit ook als HEP?

Dit is een goede toevoeging en is aangepast in de HEPs van deze appendix

.

F. Classificatie van ongerief | Classification of severity of procedures

59. Kan er verduidelijkt worden wanneer cumulatief ongerief als matig/ernstig wordt beschouwd, wanneer gecombineerd met enkele 'mild'-scores?

Cumulatief ongerief is beoordeeld aan de hand van de hoeveelheid mild (of matig) ongerief die door procedures aan het diermodel wordt toegevoegd. Vandaar dat gut physiology (extra ongerief door transit/motility assays) en CRC development (extra ongerief door CT scans) een hoger cumulatief ongerief hebben dan de andere outcome parameters.

60. Welke hersenstudies (CNS) worden uitgevoerd en tot welk ongerief leidt dit?

Omdat meermaals bleek dat onze beschrijving van mogelijke linken met andere delen van het zenuwstelsel te kort schoot, hebben we besloten om de experimenten die hiertoe dienen te schrappen uit het huidige project.

61. Leiden CT-scans tot matig ongerief?

De CT scans an sich leiden niet tot matig ongerief, maar de voorbereiding die hiervoor dient wel. De dieren dienen nuchter te zijn en worden onder anesthesie gebracht waarna er een spuit in de anus moet worden aangebracht waar mogelijk ook tumoren zitten. Uit ervaring weten we dat deze procedures tot matig ongerief bij de dieren kunnen leiden.

Appendix 3 Description animal procedures

Algemeen

62. Hier komt voor de eerste maal CNS/hersenen/gedrag duidelijk aan bod. Dit is echter niet duidelijk in vorige appendices.

Omdat meermaals bleek dat onze beschrijving van mogelijke linken met andere delen van het zenuwstelsel te kort schoot, hebben we besloten om de experimenten die hiertoe dienen te schrappen uit het huidige project.

A. Experimentele aanpak en primaire uitkomstparameters | Experimental approach and primary outcome parameters

63. Is er geen WT groep (ctrl worm vs worm) tekort?

De Sox10CreERT2;TdTomato muizen zonder oral gavage van helminth wormen zijn de controles voor het wormmodel. Deze muizen zijn genoemd 'Sox10CreERT2/wnt1Cre-TdTomato (ctrl worm infection)' en staan in de 1e kolom van de non-diseased gut tabel in het document 'total number of mice appendix 3'.

64. Voor het ziektemodel, worden verschillende methodes omschreven. Echter lijkt het dat enkel het aantal dieren voor worm infectie volledig is uitgewerkt, terwijl voor andere modellen dit gegroepeerd is? Zijn aantallen voor elke methode dan hetzelfde?

Dit is inderdaad het geval.De dieren voor worm infectie zijn verder uitgewerkt, omdat dit het onderzoek is vóór het go/no-go moment, en aan de hand van dit model bepaald gaat worden welke vervolgmodellen en studies gedaan gaan worden. Het aantal dieren is bovendien afhankelijk van het experiment en daardoor de outcome parameter, en zal daarom hetzelfde zijn voor de verschillende vervolgmodellen.

65. De redenering om de go/no-go beslisstappen uit te leggen is niet duidelijk. (Als worm infectie geen effect heeft op xxx, dan doen we bv genetic editing challenge niet.) Is het niet zo dat een andere methode wel tot het gewenste effect kan leiden en de methodes dus eerder los van elkaar gezien dienen te worden?

Het worminfectie model is een veelomvattend model en, gebaseerd op eerdere studies (5.1 lid2e, 5.1 lid2h et al., 2021) verwachten we uit de resultaten afkomstig van dit model dus effectief een richting te kunnen afleiden voor het vervolgonderzoek en de modellen die hiervoor gebruikt gaan worden. Als we in het worminfectie model bijvoorbeeld een effect zien op de expressie van bepaalde genen, dan zullen we dit verder willen onderzoeken met een 'genetic editing challenge'. Als we geen specifieke targets kunnen identificeren aan de hand van het worminfectie model, zullen we ook niet overgaan naar het toepassen van genetic editing.

66. Uitleesparameters zijn wederom onvoldoende onderbouwd waarom noodzakelijk (justification), eerder een beschrijving van hoe een techniek wordt uitgevoerd.

De uitleesparameters volgen uit de background informatie van het PV en zijn daar geïntroduceerd. We hebben in appendix 3 (pagina 4) een korte algemene onderbouwing toegevoegd. In de daaropvolgende pagina's worden de uitleesparameters in meer detail beschreven.

67. Statistische methodes: zelfde opmerking als appendix 2 (12, 9, 6 dieren lijkt aan te geven dat s en d bekend zijn)

Zie antwoord appendix 2, vraag 44.

68. Bij Figuur 2 (=3?) staat: "NB – If dysregulation in the immune system, and/or the microbiome and/or ENS target genes arise from the worm infection challenge then animals can be subjected to more than one follow-up challenge (e.g. intestinal inflammation challenge and alteration in intestinal microbiota challenge)". Begrijpt de 5.1 lid2h het goed dat als bv. er door de worminfectie geen dysregulatie van het immuunsysteem optreedt, maar wel een verandering in het microbioom, u alsnog ook inflammatie wilt kunnen induceren in de dieren? Kloppen de in de figuur aangegeven no-go's dan nog wel?

We bedoelen hier dat als we meerdere effecten zien in het worminfectie model dat we deze allemaal willen onderzoeken. Dus als we een effect zien op het immuunsysteem en het microbioom, willen we deze beiden onderzoeken. Als we alleen een effect zien op het

microbioom en niet op het immuunsysteem zullen we alleen het microbioom bestuderen en dus geen inflammatie toepassen. We hebben dit proberen verduidelijken in de tekst op pagina 4 van appendix 3.

69. U geeft aan: "the provided examples of mouse models in these tables are at present the preferable models, but we will substitute them if better models become available." Kunt u aangeven aan welke criteria eventuele nieuwe modellen moeten voldoen om een van de als voorbeeld gegeven voorgestelde modellen te vervangen?

Een voorbeeld zou zijn efficientere labeling of verandering van de cellen/moleculen van interesse. Dit hebben we nu toegevoegd in het desbetreffende stuk (pagina 1, appendix 3; ook toegevoegd aan de andere appendices).

B. De dieren | The animals

70. Kunt u nader aangeven met welke varianties u gerekend hebt bij de berekening van de aantallen dieren? En wat is uw primaire uitkomstparameter?

De primaire uitkomst maten hebben betrekking op de volgende: "development/progression CRC', ENS structure, function & proliferation', 'Gut physiology', and 'intercellular communication' (zoals aangegeven in figuur 3 en in het toegevoegde tabellen document). We zullen enkele van de gut physiology experimenten combineren in eenzelfde muismodel en ook de microbiota analyse kan gecombineerd worden met 1 van de overige primaire uitkomstmaten. Wat betreft de varianties, hebben we ons gebaseerd op de experimenten uitgevoerd onder PV2017-026. We zagen hierbij dat bij het gros van de experimenten, waar we 12 dieren per groep hadden, telkens een voldoende kleine standaard error (vb 0.3 voor tumor grootte; 10-15% voor (primaire) celkweek experimenten; Figuur 1 en 2 + 5.1 lid2e, 5.1 *et al, EMBO Rep 2021).*

71. Het lijkt erop dat WT dieren binnen deze appendix niet gebruikt worden?

Dat klopt, aangezien het in deze modellen vooral het labelen van cellen/moleculen betreft. Hier willen we dus 'healthy vs disease' vergelijken binnen dit muismodel met gelabelde cellen/moleculen.

72. Welke muislijnen - en waarom deze - worden gebruikt bij go/no-go beslismoment?

Alle muislijnen zullen mogelijks een rol kunnen spelen bij het go/no-go beslismoment, omdat de labeling en activatie/inhibitie van verschillende cellen/moleculen omvat. Maar we verwachten dat de resultaten van de Sox10-CreERT2;tdT muislijn het meeste invloed hebben, aangezien dit het eerste model zal zijn wat gebruikt zal worden voor deze studies en we hierdoor een goed beeld kunnen krijgen van de effecten van het worminfectie model op het ENS.

73. U geeft aan: "we will choose specific models to continue on the other challenges/models, so we will use maximally 2 mouse lines instead of 4 for follow-up challenges". Welke selectiecriteria gebruikt u hiervoor?

We zullen hiervoor kijken naar de resultaten in het worminfectie model; in welke muislijnen we het effect waarnemen en welke 'outcome parameters' het meest interessant zijn om verder te onderzoeken in de ander modellen. Op basis van de gekozen 'outcome parameters' en bijbehorende experimenten en de effecten gezien in het worminfectie model zullen dan muislijnen gekozen worden voor deze vervolgstudies. Dit hebben we verduidelijkt in appendix 3 (pagina 8).

C. Huisvesting en verzorging | Accomodation and care

74. Welke voorzorgen dienen genomen te worden bij H poly infecties om andere muizen niet te besmetten?

De H.polygyrus is een gastrointestinale worm die alleen besmetting veroorzaakt als infectieve larven oraal worden toegediend (Camberis, Le Gros and Urban, 2003). Om deze reden worden geinfecteerde muizen in aparte kooien gehuisvest. Trigene zal worden gebruikt on de kooien schoon te maken voordat ze worden teruggezet in het IVC rek als extra voorzorg. Dit is toegevoegd in appendix 3 (pagina 9).

D. Pijn en welzijnsaantasting | Pain and compromised animal welfare

75. De 5.1 lid2h vraagt u om te specificeren welke pijnstilling binnen uw experimenten gebruikt kunnen worden. Eventuele negatieve effecten van pijnstilling op het gedrag van de dieren (bv sufheid, afvallen, etc) moeten ook benoemd worden als die een mogelijk (negatief) effect hebben op eet- en drinkgedrag en lichaamsgewicht.

Zie antwoord op vraag 57. Deze informatie is ook toegevoegd aan appendix 3 (pagina 9).

E. Humane Eindpunten | Humane endpoints

76. Dient continue diarree ook niet meegenomen te worden als HEP bij de andere inflammatie modellen?

Goede suggestie, dus dit is toegevoegd in de HEPs voor deze appendix.

F. Classificatie van ongerief | Classification of severity of procedures

77. De discomforttabellen zijn handig voor het overzicht. Hier in App3. Lijkt een behandeling/surgery te missen?: 'BAC will be surgically applied onto the serosal surface of the intestine by laparotomy (distal ileum or proximal colon)'.

De behandeling met BAC valt onder de 'inflammation/perturbed models' en is dus in de tabel opgenomen als moderate discomfort. Dit is nu ook verduidelijkt in de tekst van appendix 3 (pagina 11).

78. Kan verduidelijkt worden op basis van wat sommige combinaties (met mild ongerief) tot verhoogd cumulatief ongerief leiden dan aangegeven voor het model zelf?

Wij verwijzen graag naar het antwoord op vraag 59, aangezien voor appendix 3 hetzelfde principe geldt.

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Bijlage II:

Vragen 5.1 lid2h d.d. 19.04.2022 en Antwoorden VO d.d. 20.05.2022

Antwoordbrief 2 – openstaande vragen 5.1 lid2h m.b.t. PV2021-003

1) De pijnbestrijding in bijlage 2 en 3: hierbij geven jullie aan dat carprofen een optie is. Carprofen is echter een NSAID die een effect vertoont op inflammatie. Hoe is carprofen dan verenigbaar met de intestinale inflammatoire response die opgewerkt wordt in jullie diermodellen? Zou een bepaalde pijnscore (gaan jullie die gebruiken?) niet al snel tot een eindpunt moeten leiden met daarmee een mogelijk hogere uitval?

   De 5.1 lid2h haalt een zeer valide punt aan over de mogelijk effecten van Carprofen op inflammatie. Daarom hebben wij in de sectie betreffende analgesie enkele aanpassing doorgevoerd in appendix 2 (p. 10) en 3 (p. 9) in verder overleg met de IvD 5.1 lid2h Wij verwachten op basis van onze ervaring met deze of vergelijkbare experimenten op onze huidige PV, niet dat het gebruik van analgesie nodig gaat zijn. Desalniettemin zullen we ten alle tijden pijnscores bijhouden en als hieruit blijkt dat analgesie toch nodig is, zullen wij opiaten gebruiken in experimenten waar geen darmmotiliteit wordt gemeten of NSAIDs in experimenten waarin inflammatie geen rol speelt. Als het niet mogelijk is om analgesie te gebruiken, zullen de HEPs meteen worden toegepast.

2) De aantallen dieren app 2 en 3: jullie geven aan dat de aantallen dieren berekend zijn op basis van literatuur en uit eigen ervaring. De 5.1 lid2h zou daarom graag het verwachte verschil, de spreiding en de drop-out zien in jullie berekeningen van 6, 9 of 12 dieren per experimentele groep.

   We hebben in appendix 2 (p. 9) en appendix 3 (p. 8) een korte uitleg toegevoegd met betrekking tot de grootte van de verwachte verschillen, spreiding en drop-out. Verder zijn er enkele voorbeelden aangegeven van experimenten waar de verschillen/spreiding kleiner of groter is dan bij de andere outcome parameters. Deze aanpassingen zijn gebaseerd op literatuur en eigen ervaring met deze experimenten.

3) Het is de 5.1 lid2h onduidelijk waarom CT beeldvorming dient te gebeuren bij nuchtere dieren, als antwoord (vraag 61) waarom CT beeldvorming matig ongerief omvat. Het nuchter zijn van de dieren staat niet omschreven in de procedures, maar is wel extra ongerief en bovendien afwijkend van de 'standaard verzorging'.

   De CT beeldvorming wordt toegepast bij nuchtere dieren omdat stoelgangpellets anders kunnen interfereren met de zichtbaarheid van de tumoren in de dikke darm. De dieren zullen echter maar een korte duur (timing afhankelijk van het dag-nacht ritme) voor het maken van de CT scan nuchter gezet worden, en onmiddellijk na het maken van de CT scan zal het voer weer worden toegevoegd aan de kooien. De dieren zullen hiervan geen extra ongerief ervaren, waardoor het cumulatieve ongerief van dat de dieren ondergaan ook niet zal veranderen. Het nuchter zetten van muizen is nu ook in de tekst van appendix 2 toegevoegd (p. 5+9).

4) De 5.1 lid2h kan geen ethische inschatting maken over het therapie gedeelte (ongerief, humane eindpunten) aangezien wordt aangegeven dat dit afhankelijk is van de uitkomst van pilootstudies die nog dienen te gebeuren op het huidig lopende PV (en daarom nu als maximaal ernstig wordt ingeschat). Echter op vraag 38 geeft u als antwoord dat alle therapie studies van huidig lopend PV niet meer zullen uitgevoerd worden en daarom zijn meegenomen in dit PV. Dit lijkt dus een belangrijke tegenstrijdigheid te zijn. Bovendien is het feit dat dieren ernstig ongerief kunnen ondervinden een belangrijk ethisch dilemma in deze aanvraag. Daardoor kan de 5.1 lid2h

5.1 lid2h over dit gedeelte nu geen beslissing nemen. We stellen daarom voor om OF deze pilootstudies mee te nemen in huidige aanvraag met een duidelijke go/no-go beslissing naar de eigenlijke therapie studies (inclusief zo veel mogelijke beperking van het ongerief) OF de therapie-studies uit de aanvraag te halen.

Zoals correct opgemerkt zullen enkel de pilootstudies, maar niet de officiële therapiestudies uitgevoerd worden op het lopende PV. Deze huidige PV loopt namelijk nog 1,5 jaar, waarin wij verwachten deze pilootstudies uit te voeren. Daarom zijn deze pilootstudies dus niet meer opgenomen in deze nieuwe PV aanvraag. De therapiestudies zelf krijgen we niet meer uitgevoerd in de komende 1,5 jaar, waardoor deze wel opgenomen zijn in deze nieuwe PV. Om ervoor te zorgen dat er een goede ethische overweging gemaakt kan worden hebben we nu een duidelijker go/no-go moment toegevoegd (appendix 2, p.5). De pilootstudies uit de lopende PV zullen als go/no-go moment gelden voor de therapiestudies in deze PV. In het geval het niet mogelijk blijkt (op basis van de pilootstudies) om de therapiestudies met matig ongerief uit te voeren, zullen deze experimenten vervallen (max 432 dieren). Het ongerief zal hierdoor zoveel mogelijk beperkt worden en wordt geschat op moderate.

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
1.3	Provide the title of the project.	The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease

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.

The **gastrointestinal tract** is built from a complex set of tissue components and cellular networks of distinct embryological origin that integrate their activity to continuously control gut function. This structure extends from the oral cavity to the rectum and performs essential roles such as digestion and absorption of nutrients and water, motility, and host defence to maintain health and homeostasis of organisms. Because of its position at the interface between the external and internal milieu of the body, it is not surprising that the gastrointestinal tract can be a possible entry point for microbes that have been recently recognised as major contributors in host physiology (1), but also in the pathogenesis of several diseases.

Major components GI-tract

The intricate anatomy of the gastrointestinal tract includes a mucosal layer, in which epithelial cells function as a physical barrier to a highly dynamic and complex luminal environment (e.g. microbiota), and work together with the largest collection of immune cells within the body (intestinal innate and adaptive immune systems) to establish a balance between host defence (e.g. pathogens, toxins) and tolerance (e.g. nutrients, commensal microbiota). Also, the gastrointestinal tract contains an extensive local vasculature, which is essential for nutrient absorption, and acts as a gateway for endocrine signals that are produced within the gut, and ensures communication with remote sites in the body. In addition, smooth muscle layers and Interstitial Cells of Cajal (ICCs) execute distinct programs to initiate and maintain intestinal motility, which is necessary for food propulsion, digestion and waste expulsion. We firmly believe that the ***enteric nervous system (ENS)***, the system of interest of our lab, works as the central hub that tunes this multifaceted set of chores to maintain gastrointestinal homeostasis (2).

The Enteric Nervous System: development, organization and functions

In mammals, the ENS is derived from neural crest cells that migrate throughout and colonise the entire length of the gastrointestinal tract. By receiving adequate inputs, ENS progenitors also migrate centripetally from the muscle layers towards the mucosa, colonising and differentiating into more mature and functional enteric glial cells (EGCs) and neurons that orchestrate most aspects of gastrointestinal function, including mucosal absorption and secretion, intestinal blood circulation, host defence and motility (3, 4).

Given the critical steering of ENS functioning to maintain gastrointestinal homeostasis, it is not surprising that developmental (i.e. congenital) or environmental (i.e. acquired) deficits in the ENS have been linked with severe neuropathies such as Hirschsprung's disease, and functional gastrointestinal disorders such as irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD) (5). In addition, the role of nerves in (e.g. colorectal) cancer is an emerging research field, as neurotrophic factors derived from enteric neurons have been shown to act in the development and progression of several cancer types (e.g. stomach) (6). In this context, we recently uncovered a role for the NDRG4 gene, one of the most accurate biomarkers for colorectal cancer (CRC) (7) which is specifically expressed in the ENS (8), in both ENS development and colorectal pathogenesis. In fact, ENS development and intestinal functioning is negatively affected upon knockdown of *ndrg4* in zebrafish (9) and lack of *Ndrg4* in mice alters enteric neuronal signalling and aggravates the progression of CRC (10). Furthermore, both published **(Figure 1)** and unpublished data from our lab **(Figure 2)**, suggest a role for the ENS in the biology of CRC and that these cells can affect/communicate with each other (8, 10) which has to be studied further. Based on this, cancer therapy might have different effects when the ENS is altered, which is also an area of interest for our lab.

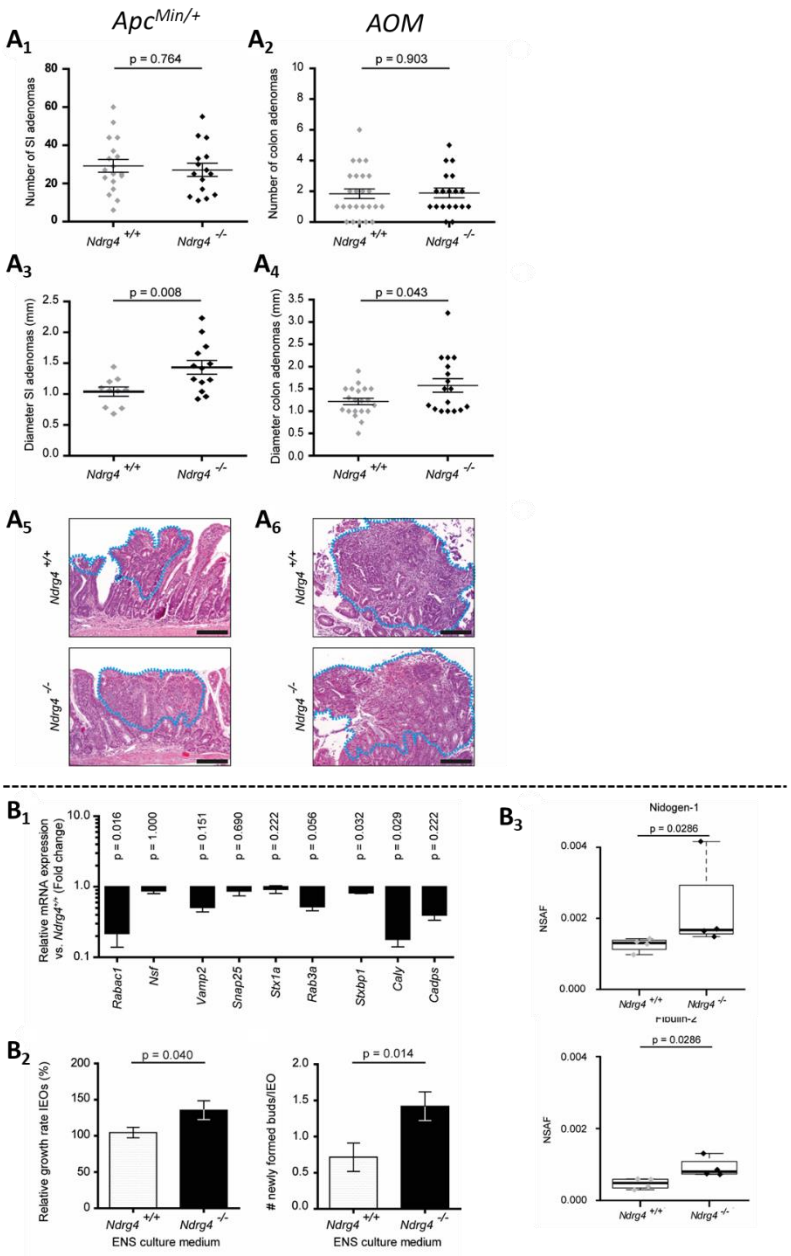

Figure 1: *Loss of enteric neuronal Ndrg4 is associated with enhanced CRC progression, most likely via affecting vesicle trafficking and the increased release of two extracellular matrix proteins (Nidogen-1 & Fibulin-2).* **(A)** Whereas loss of *Ndrg4* has no influence on the incidence of small or colonic adenomas **(A_1-A_2)**, it correlates with enlarged **(A_3-A_4)** and more aggressive **(A_5-A_6)** adenomas. **(B)** Mechanistically, primary ENS cultures characterized by loss of *Ndrg4* have revealed alterations in the expression of genes involved in vesicle trafficking **(B_1)** and medium derived from these cultures significantly enhances the growth of intestinal epithelial organoids **(B_2)**. Mass spectrometry on this culture medium uncovered the increased levels of two extracellular matrix molecules Nidogen-1 and Fibulin-2 **(B_3)**.

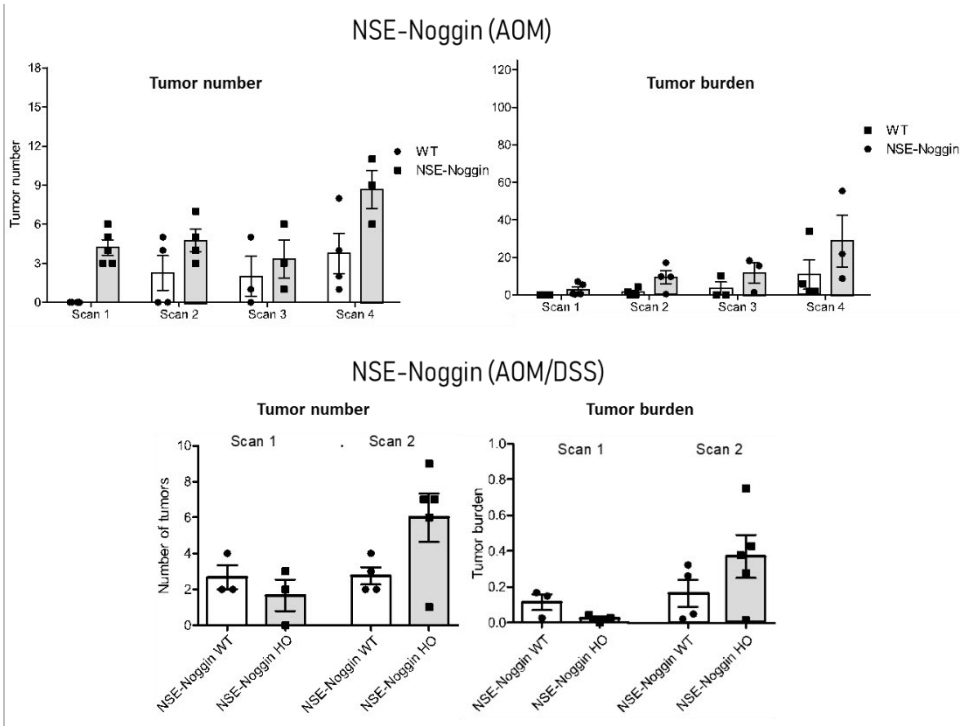

Figure 2: *CT scan results from pilot experiments of mice with normal number of neurons (WT) or more neurons (NSE-Noggin) in the ENS that underwent either AOM CRC induction protocol of AOM/DSS CSC induction protocol.* NSE-noggin (AOM): mice with more neurons seem to develop tumours faster and have a higher tumour burden at end stage than WT littermates. NSE-Noggin (AOM/DSS): onset of cancer seems to be similar, but tumour number and burden seem to be increased in mice with more neurons compared to WT.

Although the ENS was first described many decades ago and considerable progress has been achieved in recent years understanding this system, there are still several challenges to overcome in mammals. Novel insights emerging from the transcriptional profile and organisation of enteric neuronglia units have shed light on the developmental principles that govern the assembly of the ENS (3, 11, 12). However, despite many similarities in the sophistication of neural circuits with other parts of the nervous system in vertebrates, the topographic organisation of the ENS cells within the gut wall is seemingly chaotic and very complex. Neuronglia units contain distinct neuronal and EGC subtypes that are arranged into two concentric and interconnected structures called plexuses, forming in a complex and heterogeneous wiring network within the intricate setting of the gut wall. In recent years, our and other laboratories worldwide have put an extensive effort to develop high-resolution techniques to identify and label ENS cells in order to map and understand the organisation of ENS components under physiologic and pathological conditions (1, 3, 11-17). Nevertheless, our understanding of **how ENS signalling arises from the mature neuroglial circuits to connect to intrinsic and extrinsic intestinal tissues (e.g. epithelial, immune cells, vasculature, microbiome and brain) lags far behind.**

The ENS partners with microbiota and the brain

The ENS functions partially independently of inputs from the central nervous system (CNS), yet it is a pivotal relay system in the bidirectional communication between the gastrointestinal tract and the brain (i.e., gut brain axis). The physiological importance of this microbiota-gut-brain axis has been highlighted by published work from our group and collaborators, revealing that tissue and behavioural defects on the ENS are associated with alterations in the microbial landscape (1). For instance, by combining genetic tools and novel strategies to label and isolate intestinal intrinsic neurons, we demonstrated in a collaboration with the Francis Crick Institute (London, UK) that microbiota regulate the physiology of the ENS. This study highlighted the consequences of tissue and environmental changes on ENS behaviour (from alterations in ENS transcriptomic landscape to the interruption of normal performance of tissue physiology) (1). Furthermore, in collaborative work with other groups at 5.1 lid2h , we have recently shown that maternal exposure to intestinal pathogens during pregnancy alters the levels of markers that are expressed exclusively by ENS cells in the foetus, and results in increased infiltration of immune cells (18). Altogether, work from our group and other (collaborative) laboratories proposes that the ENS is crucial in maintaining the normal physiology of the gastrointestinal tract by interacting with other tissues and systems. More importantly, these studies emphasize the plastic potential of the ENS as it responds to intrinsic (tissue) and extrinsic (environmental) cues that affect the gastrointestinal homeostasis. **However, the field still lacks on specific mechanisms by which ENS cells communicate with other systems to maintain tissue integrity or respond to insults.** Interestingly, we have recently found that EGCs upon helminthic infection with Heligmosomoides polygyrus (H.

poly), significantly upregulate molecules that are involved in the production of extracellular vesicles (EVs; e.g., CD63, CD9) (5.1 lid2e, 5.1 lid2h

These data **suggest** a putative role for EGCs-derived EVs in enteric cell-to-cell communication e.g. by carrying epitranscriptomic modulators such as miRNAs (Holland et al., unpublished observations) in disease progression and/or resolution.

Consequently, our project **aims to** develop a holistic and advanced approach to study the influence of the intestinal environment on ENS transcriptome, morphology and physiology, both in health and disease. More specifically, we want to further **investigate how the ENS responds to physiological challenges in the luminal environment (e.g., microbiota) and to local pathologies (e.g., cancer and helminthic infections)**. At the same time, we will assess how alterations in ENS homeostasis (e.g. caused by microbial changes) influence other parts of the nervous system (e.g. central nervous system/ peripheral nervous system, as part of the gut-brain axis).

To achieve and succeed in this project, we are committed to apply well established *in vitro* analysis of ENS cell types and intestinal organoids to reduce animal use. We have confidence that all experiments performed under this project license will lead to a better understanding of the adult ENS. We believe that any progress in understanding the spatial organisation, the molecular landscape and the function of the ENS, as relevant as it will be, unravels only small fragments of the gut biology. Therefore, and to better comprehend the aetiology and the consequences of enteric neuropathies and other diseases that affect gastrointestinal function, we will have to apply integrative approaches that take into account the dynamic interactions between the ENS surrounding intestinal tissues and the luminal microenvironment.

Part of the research described in this PL (mostly concerning appendix 2) will follow up on the research carried out under our previous project license 2017-026. The data of these experiments demonstrate that the mouse lines and CRC disease models are appropriate for studying the same outcome measures in other mouse lines and new outcome measures on both already in use and new mouse lines (see Figure 1 and Figure 2 above). Because we see an effect of ENS-specific NDRG4 and enteric neuron density on CRC development/progression (Figure 1 and 2), we want to continue studies in these models to get further mechanistical insights into this effect and study the effect on CRC therapy. Furthermore, we want to follow this research concerning enteric neurons in CRC by studying enteric glia as they are the other important cell type from the ENS that is present in the tumour microenvironment.

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

The **ultimate goal of this project** is to get a better understanding of the molecular pathways that orchestrate the organisation and function of the ENS, and its integration with other tissues (e.g., brain) and systems (e.g., immune, vascular and epithelial systems) during physiological challenges (e.g. microbiota) and pathological challenges (e.g. gastrointestinal diseases like cancer, worm infection). This will bring us closer to understand how the ENS participates in the onset and progression of diseases that affect the gastrointestinal tract, and therefore will eventually contribute to the development of new targets and therapies to tackle gastrointestinal disorders. For this purpose, we aim to develop a holistic and advanced approach to study the influence of the intestinal environment on ENS transcriptome, morphology and physiology, both in health and disease, for which we will use well-established murine models and procedures.

It is important to state that this project license will work as an umbrella to cover 4 parallel PhD projects in our group. Therefore, the main question in this project proposal will be minutely investigated by addressing 3 sub-aims (= immediate goals), which are described below.

Aim 1: Generate and use *in vitro* (co- culture) systems to study cell-to-cell communication (Also used to address aims 2, and 3) – Appendix 1

Because studying communication between different systems/cell types *in vivo* is difficult, we will generate and use *in vitro* systems to study mechanistical insights dictating cell-cell communication and use this information to increase the likelihood of success in aims 2 and 3 (e.g. by targeting/labelling/studying specific cells/vesicles/molecules that we found here in aim 1). We will assess potential communication mechanisms like EVs and miRNAs in (in-)direct co-culture models using different (primary) cell types like ENS cells versus CRC (co-cultures with available CRC cell lines), intestinal

organoids, epithelial and/or endothelial cells (see Strategy section for detailed explanation and justification). Importantly, whenever possible, preliminary data will be acquired by using *in vitro* systems (both cell lines and primary cell cultures, see appendix 1) prior to experiments that might cause long lasting harm, pain and distress to experimental animals *in vivo*.

Aim 2: Investigate cellular and molecular mechanisms of interaction between ENS, intestinal (cancer) and other cells in a cancerous environment – Appendix 2

Both published (Figure 1) and unpublished data (Figure 2) from our lab, suggest a role for the ENS in the biology of CRC and that the ENS can affect/communicate with other intestinal cell types (8, 10). The CRC-related research described in this PL follows and elaborates on our findings and ideas obtained with the previous PL (AVD5.1 lid2h), as described in detail in appendix 2. With these experiments, we aim to further delineate the participation of EGCs and neurons on the onset and progression of CRC, the effects of their interactions on both CRC cells and ENS cells and the possible mechanisms involved in this interaction (see Strategy section for detailed explanation and justification). Further explanation of read-outs and techniques is described in the Strategy section and Appendix 2.

Aim 3: Investigate the mechanisms of cell-to-cell communication in homeostasis and diseases that affect the gastrointestinal tract (e.g., worm infection, (and/or) microbiota alteration, (and/or) inflammation)- Appendix 3

Preliminary data from our lab and collaborators suggests that the infection model with *H. poly* is ideally suited to address the research question in aim 3, as it affects gastrointestinal homeostasis and triggers an ENS response important for disease resolution. However, the latter needs further investigation. After acquisition and analysis of data using this model and depending on the outcome/mechanisms or cell types that seem to be involved, we will decide on the road to further investigate other gastrointestinal challenges (e.g., inflammation, microbiota manipulation) to confirm whether the ENS phenotype and involved molecular pathways observed in response to *H. Poly* are shared amongst other gastrointestinal diseases. Further specification of this and explanation of read-outs and techniques is described in the Strategy section and Appendix 3.

The experiments performed under this project license are aimed to dissect the pathways by which the ENS interacts with other cellular systems in health and disease. We will investigate e.g. the role of ENS cells, ENS-derived EVs and miRNAs in controlling gastrointestinal function, disease progression and resolution *in vitro* and *in vivo*.

3.2.2 Provide a justification for the project's feasibility.

All experiments suggested above can be successfully performed during the course of this project as the expertise and facilities needed to execute them are available, either in the host or in other laboratories, with whom we have established solid collaborations. In fact, the host lab has been able to establish optimized protocols for the successful generation of all primary cell types and (in-) direct co-culture models with the previous PL. Also, plenty of expertise regarding the different CRC and CRC-treatment models have been gained with the previous PL. Expertise regarding the other models have been gained by the collaborative labs, who are currently still advising and helping us with respect to these models.
Although it will be the dominant topic of interest of our laboratory, our work will not only include analysis on the ENS but will also represent contributions from other groups/institutes that have expertise on other non-ENS systems (such as immune system, CNS, other cell types present in the gut), interests, reagents and materials, wildtype or genetically manipulated animal models, to fully integrate the foundation of Science, and improve the impact, relevance and reproducibility of the experiments adhering the premises of the 3Rs.

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.

3.3 Relevance

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

Relevance for research: Research interest focusing on the gut has increased in recent years because of the clinical and biological relevance of this organ in several diseases. More specifically, it has been recently shown (by our and other groups) that the ENS interacts with multiple systems and has been implicated in the onset and progression of many diseases that not only affect the gastrointestinal tract but also the CNS (5). Due to the intricacy of its network and close proximity with many other tissues, studying the nervous system of the gut is challenging and requires expertise from distinct fields. Our group and collaborators have all necessary tools and skills to thoroughly dissect the cellular and molecular pathways that underlie the ENS crosstalk with other systems in various conditions. Given the similarities in composition, organisation and function between the ENS and the CNS, investigating the role of the ENS in homeostasis and disease would allow scientists to take the complexity of the gut to study other organs. We expect to unite different fields to unravel many other biological questions raised in this project and contribute for the consolidation of enteric neuroscience.

Relevance to the patient: Increasing evidence shows that the ENS might be the “entrance door" for several pathologies, including those affecting brain homeostasis. In neurodegenerative diseases, for instance, ENS phenotype and gastrointestinal malfunction have been shown to precede brain and/or motor symptoms by several years (5). Moreover, the ENS is likely to contribute to carcinogenesis, as both neo-neurogenesis and perineural invasion are unfavourable factors for CRC patients, which indicates that their survival rate is negatively affected by the higher nerve density in the tumour area (i.e. neo-neurogenesis) and invasion of tumour cells throughout nerve fibres (i.e. perineural invasion) (6). With confidence, it is conceivable to suggest that the participation of the ENS in diseases that affect the gastrointestinal tract and systems beyond it (i.e., gut-brain axis) deserves more attention.
We aim to study whether the ENS functions as a key player in the maintenance of the fitness of the intestinal microenvironment, and its crosstalk with the brain. Furthermore, we consider the ENS as a potential target for therapies in diseases that affect the homeostasis of the gastrointestinal tract in humans. Our prospect is thus to unravel the role of ENS behaviour in homeostasis and under circumstances that disturb the equilibrium of the healthy gastrointestinal tract. Therefore, we will include relevant animal and *in vitro* models to mimic human conditions, (e.g., cancer and helminthic infections), that impair intestinal function. By using advanced technology to study the ENS and powerful insights from experts in gastroenterology, we intend to identify novel cellular and molecular mechanisms, and biomarkers (e.g., EV-derived small molecules, non-coding miRNAs) that translate the onset of diseases progression and/or (response to) treatment.

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

The project stakeholders include the mice, the scientific community - in the same and related research areas- and ultimately patients, physicians and the pharmaceutical industry. The scientific community will immediately benefit from our findings. Exploring the basic mechanistics described within this PL allows us to identify new pathways and targets, which will ensure that our understanding of these diseases is improved. Besides, these fundamental insights might have implications for related diseases. Results will be published within peer-reviewed journals making them available to the entire scientific community. This ensures that our group will be identified as “experts” within this research field and allows other researchers to take advantage of these insights to further explore mechanisms and develop therapies. Long-term benefits are for patients, clinicians and health systems as our research will delineate new insights in the different disease mechanisms. Also, the pharmaceutical industry will benefit from this research project in case we find putative and novel cellular targets in diseases that alter the homeostasis of the GI tract. Finally, the mice represent the only stakeholders that do not immediately benefit from this project. Despite potential discomfort, these stakeholders are vital for this PL because the represented *in vivo studies* will allow us to gain new insights which will eventually ensure that all other stakeholders benefit from this project. On the other hand, we will try to minimize the need for *in vivo* studies with discomfort as much as possible by generating and using the *in vitro* models (aim 1).

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 study our main goal “the cell-to-cell communication between the ENS and intestinal and extra-intestinal tissues in health and disease” we have formulated the following specific aims and sub-aims:

1. **To generate and use *in vitro* systems to study cell-to-cell communication (Appendix 1),** we will derive cells from murine intestines and use these in *in vitro* analysis to assess the communication between the ENS and other cell types. These model systems will also allow studies of intracellular signalling mechanisms involved in intercellular

communication and this information will be used to target/label/study for example specific cells/vesicles/molecules to address aims 2 and 3. Therefore, the *in vitro* studies in this appendix can inform us about which outcome measures to study, what results to expect or which molecules/cells to target in the *in vivo* studies of appendix 2 and 3. Furthermore, results from the studies in appendix 2 and 3 can lead to the need for specific mechanistical or adaptational studies in this appendix, because they can only be done *in vitro*. More information on the numbers of animals/cell types and read-outs are described in Strategy below and appendix 1.

2. **To investigate cellular and molecular mechanisms of interaction between ENS, intestinal (cancer) and other cells in a cancerous environment (Appendix 2),** we will study the role of enteric neurons and EGCs in the onset and progression of CRC.
   Aim 2a: The role of EGCs in the development, progression, and treatment of CRC
   Aim 2b: The role of enteric neurons in the development, progression, and treatment of CRC
   More information on the numbers of animals and read-outs are described in Strategy below and appendix 2.
3. We will **investigate the mechanisms of cell-to-cell communication in homeostasis and diseases that affect the gastrointestinal tract (e.g., worm infection, (and/or) microbiota alteration, (and/or) inflammation) (Appendix 3)** to establish the role of ENS-derived molecules (e.g., EVs, miRNAs) to maintain the fitness of the gastrointestinal tract *in vivo*, and in the progression and resolution of diseases that affect the gut. The first model of choice is the *H. Poly* (worm infection). In this model we will study which phenotype will arise. Depending on whether dysregulation in the immune system, and/or the microbiome and/or ENS target genes arise, then animals can be subjected to microbiota alteration and/or inflammation (see Figure 4, Go/no-go moment).
   Aim 3a: The role of ENS-derived EVs for cell-to-cell communication in health and disease.
   Aim 3b: The role of ENS-derived miRNAs
   More information on the numbers of animals and read-outs are described in Strategy below and appendix 3.

To study the above-described aims and sub-aims, we will use wildtype mice and mice with altered ENS **(explained below in 3.4.2)** that are characterised by fluorescently labelled* (ENS) cells and are either unchallenged (i.e., non-diseased gut) or challenged with various stimuli to mimic or elicit certain diseased conditions (e.g., colorectal cancer, worm infection, alterations in intestinal microbiota…).

**Fluorescent labelling (including activity reporters such as genetically-encoded Ca^{2+} indicators) of the cells in the murine models will either be achieved by crossing mice that are (epi)genetically altered to drive expression in a cell-specific manner (based on promotor activity), with mice harbouring transgenes for conditional fluorescent reporter or by labelling cells by means of viral vector (e.g AAV) transduction.*

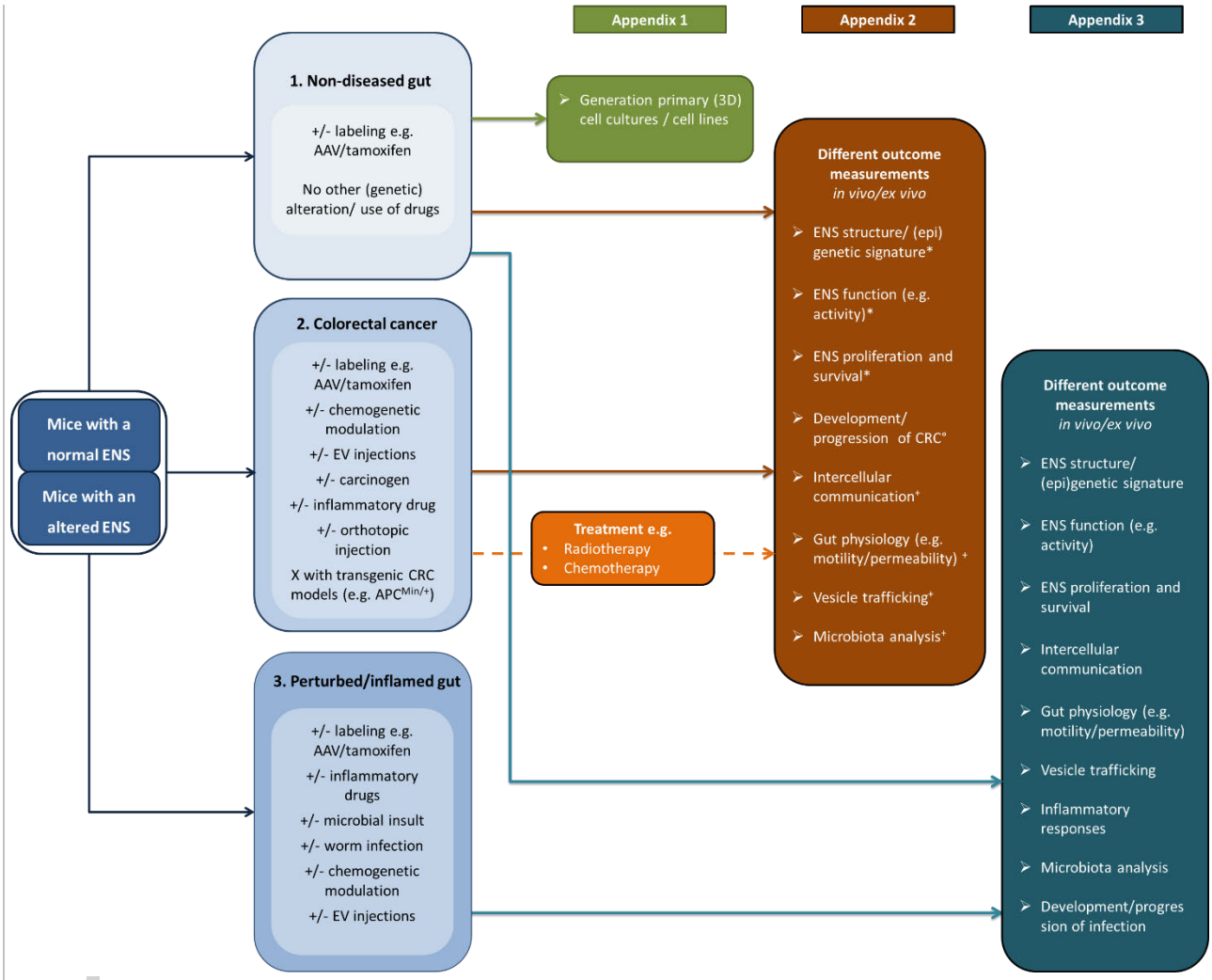

Figure 3. *Flowchart that will be followed over the duration of this project license, depicting the working model for the wild-type and transgenic murine models that will be used to examine the above-described goals. Note: *, +, ° behind the outcome measures of appendix 2 correspond to one of the green blocks in Figure 3 used to specify the strategy for this aim.*

In appendix 1 (Figure 3, green colour), we will generate and use cell culture models (e.g., ENS cells, neurospheres, 3D cultures) derived from the intestines of mice with a normal ENS and from mice with an impaired ENS to examine the communication between the ENS and other cell types *in vitro* (max 10 cell-cell interactions such as enteric neurons and enteric glia cells with macrophages, smooth muscle cells, epithelial cells, cancer cell lines) and intracellular signalling mechanisms involved in intercellular communication (max 7 signalling pathways such as calcium signalling, different extracellular vesicles (e.g. CD63+, miRNAs) (justification of these interactions and signalling pathways can be found within the background section: Major components GI-tract (p4) and The ENS,: development, organization and functions & The ENS partners with microbiota and the brain (P2-4), respectively). If available, established (secondary) cell lines will be used prior to any use of animals for culturing primary cells.

- **Animal models:** Wildtype mice, mice with labelled/activity modulated ENS cells and molecules, and mice with a (genetically) altered ENS;
- **Animal procedures:**

We will isolate murine gastrointestinal tracts from mice models with labelled cell types and/or an altered ENS (e.g. cell numbers, activity or miRNA expression) to generate homogeneous and mixed primary (3D) cultures (e.g., intestinal cells, EGCs, neurons and progenitors, and organoids). These different cell types will be used in *in vitro* experiments to study the interplay between the ENS and gut functioning in the non-diseased gut.

To investigate the role of ENS cells (e.g. vesicle secretion/ENS activity/miRNA) in the gut and their interactions with epithelial/immune/cancer cells (co-cultures with available cell lines), animals from the different mouse lines will be sacrificed after weaning for the generation of primary, mature (3D) cell cultures. In case of culturing progenitor cells and neurospheres, neonate mouse offspring will be used.
The animals may be subjected to administration of substances (e.g., tamoxifen, BrdU) and viral particles (e.g., xAAVs) to induce labelling (e.g. fluorescent reporter) or modulation of ENS cells and/or mark cellular processes (e.g. cell proliferation, cell death).
We will have to isolate all cell types from each model independently, because specific cell types need to be labelled and/or changes in the ENS need to be induced. Answers derived with either of these models may provide some useful insights that can be used/applied in the other models, but will definitely not provide answers we aim to address with the other models. Thus, given that all models serve their own purpose and will be used to answer specific questions, we cannot specify specific go/no-go moments within this aim.

In appendix 2 (Figure 3, orange colour), *in vivo* and *ex vivo outcomes* (listed below) will be investigated in our murine models of colorectal cancer compared to non-diseased gut and/or different genotypes that affect the ENS compared to each other in a CRC model. These CRC models are described in more detail in appendix 2. Below and in figure 3, we have defined 8 outcome measures, which will allow us to investigate the interplay between the ENS and CRC cells, and how they affect other cell types that are part of the tumor microenvironment, such as immune cells and microbiota, which will be studied along the ENS-CRC interaction (justification for these measures follows from the background section):

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)*
- ENS function (e.g., ENS connectivity and network activity)*
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)*
- Development/progression of CRC (e.g., track tumour burden)$^{+}$
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)°
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)°
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)°
- Microbiota analysis (e.g., composition and abundance, bacterial secretome)°

*Note: *, $^{+}$, ° correspond to one of the green blocks in Figure 4.*

- **Animal models:** Control mice and mice treated with carcinogenic/inflammatory agents or crossed with the APC$^{Min/+}$ mouse model and/or mice with labelled/activity modulated ENS cells or molecules and/or mice with altered ENS;
- **Animal procedures:**

Here, we aim to investigate and unravel the interface of the adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota) in health and colorectal cancer using well-established cancer induction protocols (e.g. AOM, AOM/DSS) or genetically induced cancer. Hereby, we will explore the interaction between the ENS and CRC and the possible mechanisms involved in this interaction based on the procedures/outcome measures described above (maximum of 8 outcome measures to study all interactions and mechanisms). In addition, after euthanization of the mice, we will isolate tumours and adjacent normal epithelium, to generate intestinal (tumour) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact mechanism by which the ENS affects the epithelial and tumour cells and vice versa. In case of success in acquisition and analysis of data using these models (go/no-go), we will also study the potential impact of an impaired ENS (genetically/chemically-induced alteration) on current treatments of CRC (e.g. radio- and chemotherapy).

We will use these different mouse lines, CRC disease models and procedures to characterize the ENS and the interplay with CRC cells and other cell types that are involved in these processes.
Because this research is mainly set up to properly characterize the cell types, processes and pathways involved, it is difficult to clearly specify Go/No-go moments as this would limit the progress of the characterization process. However, in the figure below, we tried to clarify and structure our research strategy for this aim using decision moments where possible (**Figure 4**). Based on experience derived from the experiments in AVD5.1 lid2h , we know that most of the disease models work sufficiently to induce CRC, so these models can be immediately applied to study all outcome measures in detail. To start with, the chemical cancer induction models: AOM and AOM/DSS are the models of choice (specifics about the models can be found in appendix 2). However, we want to use the genetic CRC APC model or an orthotopic CRC model to answer specific questions e.g. labelled cell tracking, mechanistic insights human CRC process. After analysis of the gained CRC data based on changes in cancer development/progression (outcome measure identified with $^{+}$ above),

we will know whether it is worthwhile (e.g. sufficient effects of the ENS on CRC process) to apply chemo/radio therapy (Go/No-go, Figure 4 left). After analysis of our outcome measures identified with a * above that study ENS structure, function, proliferation and/or activity, we will decide on the need (e.g. sufficient effect of CRC on the ENS) for further functional studies using the other outcome measures identified with a ° above (Go/No-go, Figure 4 right).

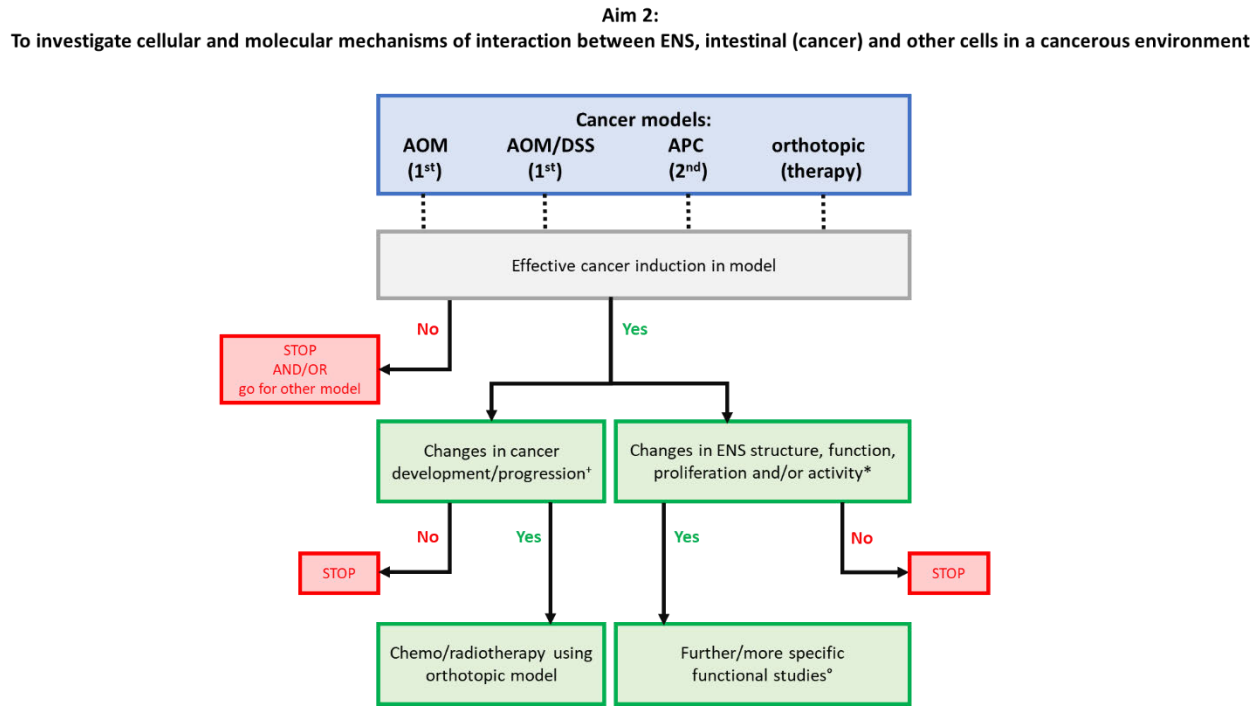

Figure 4. *Flowchart depicting crucial Go/No-go decision moments in appendix 2. *, +, ° in the green boxes correspond to the outcome measures described above and in Figure 3 (see above).*

Note 1 - Although experience gained with PL 2017-026 reveals that the different cancer models are efficient in several of the models that we will also use in this PL (e.g. Ndrg4, Hand2fl, NSE nogging) we are currently still unsure about the effectiveness in all other models that will be used in this PL. Also, given that we will have to re-order the APCmin/+ mice as we do not have them available within our facility anymore, we do not know if the efficiency of spontaneous cancer development will be similar as to what we have observed previously.
Note 2 – The "Further/more specific functional studies" are specified in detail within appendix 2 and entail the outcome measures identified with a + in the text above. Data derived from these experiments will provide further insights into the functional mechanisms that are of importance during cancer development and will enhance our understanding of colorectal pathogenesis.

In appendix 3 (Figure 3, dark blue colour), *in vivo* and *ex vivo outcomes* (listed below) will be investigated in our murine models of inflammation or other altering physiological cues compared to non-diseased gut (maximum 4 different models) and/or different genotypes that affect the ENS compared to each other in a disease setting (maximum 4 different genotypes for the 1st model and maximum 2 genotypes for follow-up models) (see information below and Figure 5). These models and the outcome measurements used for each model are specified in detail in appendix 3. To investigate the response of the ENS to physiological cues and inflammatory diseases that affect gastrointestinal homeostasis we will focus on maximum 10 different outcome measurements (further justification for these outcome measures follows from the background information):

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)
- ENS function (e.g., ENS connectivity and network activity)
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)

- Inflammatory processes (e.g., immune cell phenotype)
- Microbiota analysis (e.g., composition and abundance, bacteria secretome)
- Development/progression of infection (e.g., track parasitic infection and worm burden, numbers and eggs)

- **Animal models:** Wildtype mice and mice with labelled/activity modulated ENS cells or other molecules (control vs perturbed/inflamed gut).
- **Animal procedures:**

Here, we aim to investigate and unravel the interaction between adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota) in health (1) and/or local and systemic challenges, such as worm infection (H. poly), (epi)genetic editing (AAVx-carrying genetic modifiers), and/or intestinal inflammation (DSS or BAC), microbiota alterations (depletion/antibiotics), chemogenetic modulation (DREADDs) (further justification for these models follows from the background information). The same lines can be further examined by rescue experiments to re-establish the intestinal microbiota composition (reintroduction of microbes by faecal transplantation), rescue of specific phenotype by using advanced technology to deliver molecules (AAVx.transgene).

The *H. Poly* (worm infection) model will be first used to study this aim as it affects gastrointestinal homeostasis and triggers an ENS response important for disease resolution. If dysregulation in the immune system, and/or the microbiome and/or ENS target genes, arise from the worm infection challenge, then animals can be subjected to one or more of the follow-up challenges separately (e.g. intestinal inflammation challenge, genetic editing and alteration in intestinal microbiota challenge). These challenges can subsequently be used to study underlying mechanisms for the dysregulation of the systems/cells found in the *H. Poly* model (maximum 10 different outcome measures). Figure 5 below depicts the go/no-go strategy which is further described in detail in appendix 3.

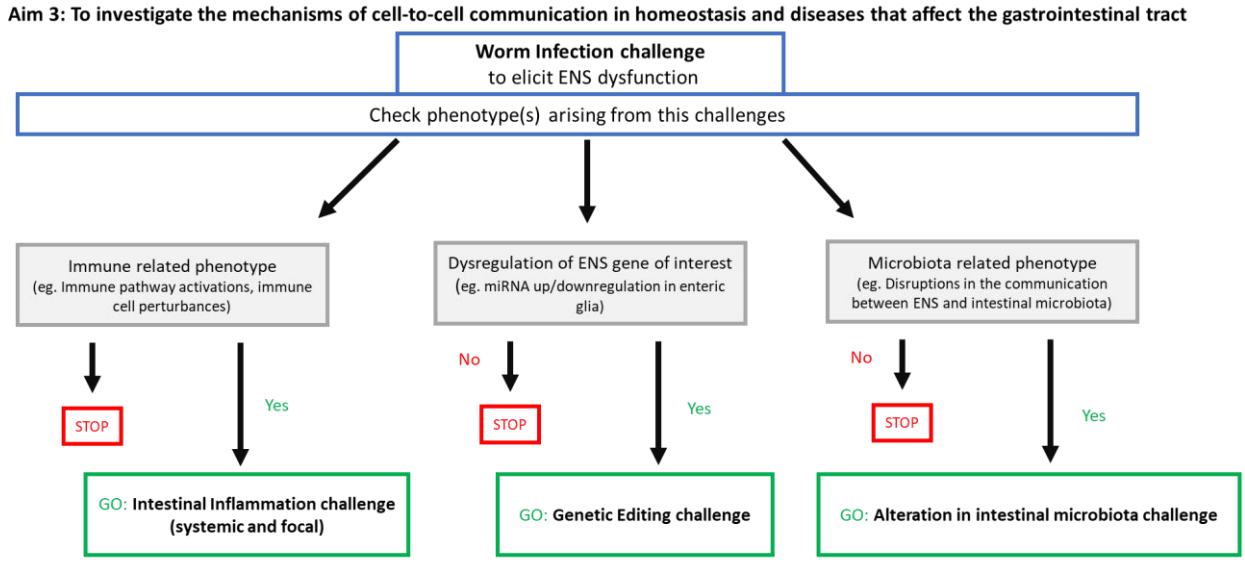

Figure 5. *Flowchart depicting crucial Go/No-go decision moments in appendix 3.*

ENS homeostasis will be analysed *in vitro* **(appendix 1),** *in vivo* and *ex vivo* **(appendix 2 and 3)** taking advantage of well-established animal models described in the respective appendices. To investigate the role of the ENS in homeostasis and disease, we will use mice with normal and impaired ENS that can be subjected to various challenges (e.g., induction of CRC, worm infection and alteration in intestinal microbiota). Outcome measures in aim 2 (appendix 2) and aim 3 (appendix 3) largely overlap as we want to study the effect/function of the ENS in both disease models (CRC vs inflammation/other perturbations). However, unique outcome measures that are specific for a disease model/perturbation are also implemented.

We will use *in vitro* assays using e.g. cell cultures whenever research questions (aim 1-3) can be addressed using these systems and prior to performing experiments in living animals if in vitro assays are available to study the outcome measure.

When *in vivo* experiments are unavoidable, we will opt for procedures that cause the least pain, discomfort and distress and the shortest lasting harm.

3.4.2 Provide a justification for the strategy described above.

In this project, we will investigate how the ENS communicates with intestinal and extra-intestinal systems and its role in the onset and progression of disease. More specifically, in order to investigate how the ENS interacts with other systems, we will take advantage of well-established mouse models (e.g., cancer (10), inflammation (10), worm infection (5.1 lid2e, 5.1 lid2h), microbiota alteration (1) and direct manipulation of the ENS itself (1) to unravel how it interacts with other cellular systems (ENS versus the immune and the vascular systems, versus the epithelial (cancer) cells, microbiota and the brain) in homeostasis and disease. To understand how one system functions, we need to acknowledge its interactions with the surrounding systems. These interactions support the idea that all progress achieved in any single tissue system, as valuable as it will be, only represents a proportion of the gut "tale", and that complete understanding of intestinal biology and gastrointestinal diseases will require integrative approaches that take into account the dynamic interactions between all intestinal tissues and the luminal microenvironment in health and disease. Hence, to have this integrative approach between the intrinsic nervous system of the gut and other non-ENS systems and identify putative mechanisms that dictate such complex gastrointestinal function in health and diseases, different animal models to label enteric nerve cells and manipulate the homeostasis of the gastrointestinal tract become essential to successfully address the enigmatic biology of ENS cells. The state-of-the-art models proposed here are necessary to 'model' the highlighted GI disturbances (e.g. CRC) and/or are needed as tools to investigate the involved molecular (e.g. miRNAs) and (patho) physiological (e.g. Ca^{2+} signalling) mechanisms.

For aim 1, all models serve their own purpose and will be used to answer specific questions. Therefore, we cannot specify specific go/no-go moments for this aim. For aim 2, we need to study different characteristics/processes/pathways that can be involved independently, however we tried to specify Go/No-go moments within out outcome measures. These outcome measures (8 categories in total) include ENS structure/ (epi)genetic signature, ENS function, ENS proliferation and survival, intercellular communication, gut physiology, vesicle trafficking, microbiota analysis and development/progression of CRC (see Strategy for more detailed explanation of these outcome measures and appendix 2 for further details). The outcome measures ENS structure/ (epi)genetic signature, ENS function, ENS proliferation and survival will be studied first, because the ENS is our system of interest (justification, see background). When changes in these outcome measures are observed in the context of cancer, further functional studies will be carried out regarding the outcome measures intercellular communication, gut physiology, vesicle trafficking, microbiota analysis to study mechanistical cues of the effect that was seen from the ENS-cancer interaction (justification for these outcome measures, see background). The outcome measure development/progression of CRC will also be studied immediately as this is our model system of interest in aim 2 (further justification, see background). After analysis of this data, we will decide on the possibility to use chemo/radio therapy if there is an effect of specific genetic models on cancer induction to see if they also respond different to cancer treatment. This will be a go/no-go moment as we will not start this study if there are no effects in the previous data (Figure 3). For aim 3, the *H. Poly* (worm infection) model will be used first and after analysis, we will decide which models will be used depending on the dysregulated system(s) found in the worm infection model. This will be a go/no-go moment and is also depicted in the figure above (Figure 5).

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	Creating and use of primary cell lines, organoids, neurospheres
2	Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer
3	Evaluating the crosstalk between the ENS and other systems on homeastasis and the onset and progression of inflammatory gut diseases
4	
5	
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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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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
1	Creation and use of primary cell lines, organoids, neurospheres

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.

As depicted in figure 1 (green box), the primary outcome parameter of this appendix is the use of homogeneous and mixed primary (3D) cultures to study cell type characteristics (e.g. morphology, proliferation, migration), activity, and cross-talk between multiple cell types. Furthermore, we will use these cultures to study the effect of alterations/modulations on the before-mentioned characteristics.

For this purpose, we will isolate murine gastrointestinal tracts from mice with labelled cell types and/or an altered/modulated ENS (e.g. cell numbers, activity or miRNA expression; details models described below the figure) to generate homogeneous and mixed primary (3D) cultures (e.g., intestinal cells, enteric glia cells (EGCs), neurons and progenitors, and organoids). These different cell types will consequently be used in *in vitro* (co-)culture experiments to study the interplay between the ENS and gut functioning in the non-diseased gut *in vitro.*

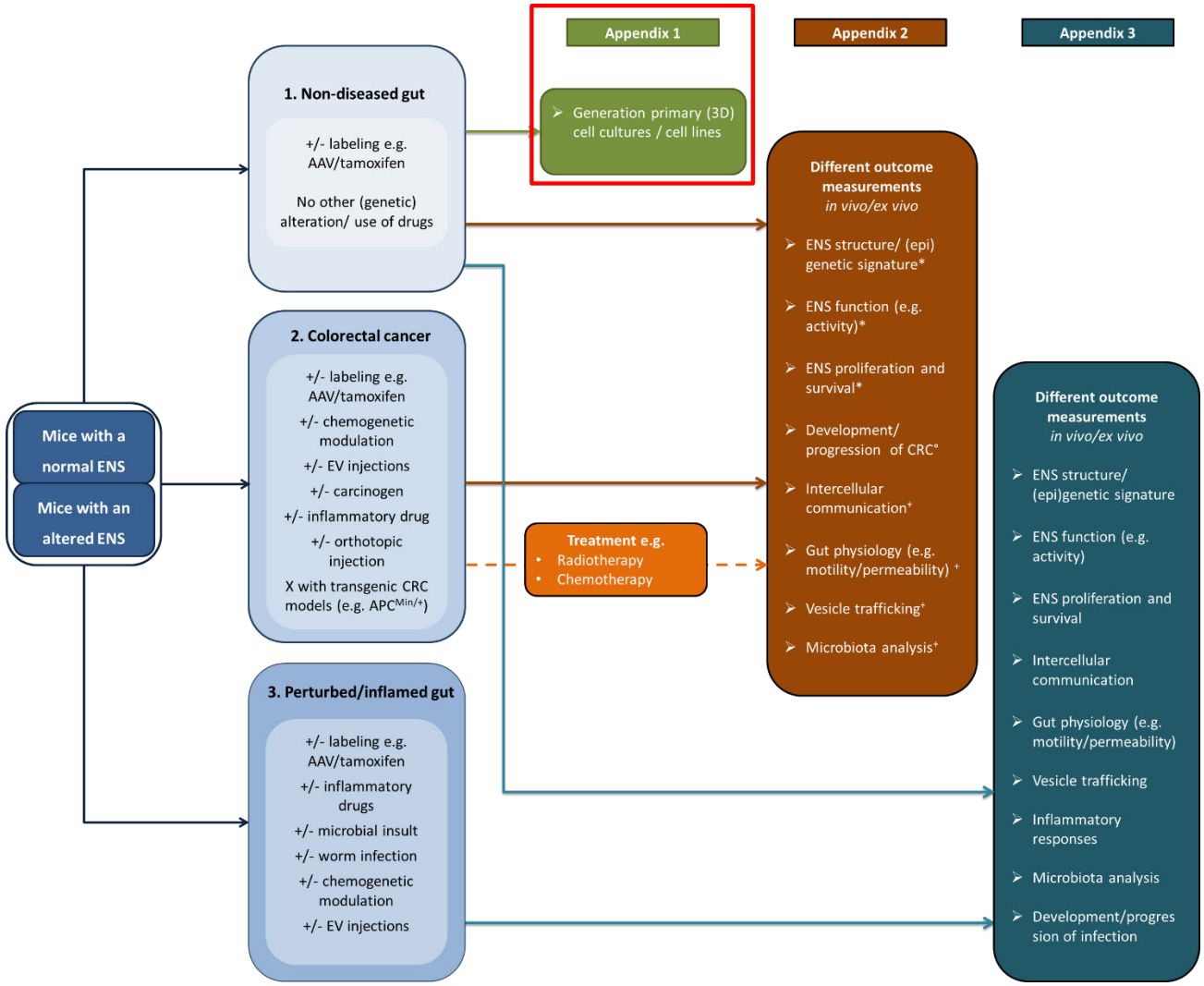

Figure 1. Outline depicting experimental models and outcome measures that will be assessed within each appendix. Red box delineates the purpose of this appendix.

Table 1: Mouse models and potential examples of mouse lines that can be used*:

Mouse model	Potential examples	Procedure
Wild-type mice		Possible saline injections Killing
Mice with fluorescently labelled ENS cells	Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}/Wnt1.Cre:R26-GCaMP6f	Tamoxifen injections (for SOX10.CreER^{T2}) Killing
Mice with fluorescently labelled extracellular vesicles from ENS cells	**CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre	Tamoxifen injections or AAV injections Killing
Transgenic mice with altered ENS	NDRG4$^{fl/fl}$Wnt1.Cre (ENS specific Ndrg4 knockdown) NSE-Noggin (more enteric neurons) Hand2$^{fl/+}$:Wnt1.Cre (less enteric neurons)	Killing
Lines designed to specifically modulate ENS activity	Sox10.CreER^{T2}/Wnt1.Cre:R26-hM4Di-DREADD	Tamoxifen injections (for SOX10.CreER^{T2}) Killing

* The mouse lines depicted within this table are at present the best models to investigate our research question. However, we would like to point out that better models might appear in the future (e.g. more efficient labelling of alteration of cells/molecules of interest). Consequently, the lines given here represent potential examples, by which means we will be able to change to

better models in case these will be designed and/or become available. However, the number of models will remain the same (justification for mouse lines under section B – genetics alterations).
**X/X = flox/flox, flox/+ or littermate wildtype controls +/+
NB - All genotypes that are bred but are not going to be used for the procedures described in Appendix 2 and 3 can be used in this appendix for culturing cells and tissues, to follow the principles of 3Rs and reduce the number of bred animals.
In the course of the experiments, we will need neonates (P1-P3) of these mouse lines for the generation of enteric neurospheres. This is further specified in the sections below.

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

To investigate the role of ENS cells (e.g. vesicle secretion/ENS activity/miRNA) in the gut and their interactions with epithelial/immune/cancer cells (co-cultures with available cell lines), offspring obtained from the different mouse lines described above will be sacrificed for the generation of primary, mature (3D) cell cultures. In case of culturing progenitor cells and neurospheres, neonate mouse offspring will be used.

The animals (after the neonate stage) may be subjected to administration of substances (e.g., tamoxifen in the Sox10.CreERT2 mice) and viral particles (e.g., xAAVs for specific outcome measures) to label or modulate ENS cells, organelles and cell events depending on the mouse line used (see table 1; applies to ~70% of mice and further explained in section B). For labelling ENS cells: 1/ when using inducible Cre recombinase (e.g. Sox10CreERT2), recombination will be induced by one or two injections of tamoxifen (intraperitoneal), 2/ one intravenous injection of xAAVs will be performed. For labelling cell cycle events (e.g. BrdU), no more than one intraperitoneal injection of substances will be performed.

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

The number of animals we will need is based on our experience regarding cell culture techniques, our newly optimized protocol and related to the number of experiments that will/can be performed per week and per year, as obtained with our previous PL (AVD5.1 lid2h).
Importantly, using our optimized protocols, we have created a consistent and reliable way to culture ENS cells, neurospheres and organoids. Based on these protocols and experience, we calculated the numbers of animals we will need to assess the outcomes of this study. These calculations are further explained below (B. number of animals).
ENS (enteric neurons, EGCs, progenitor cells), (and/or) adjacent normal epithelium will be preferentially obtained from the unused littermates coming from the breeding of the transgenic mice needed in appendix 2 and 3.

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
1	Mouse	In-house breeding	Adult & Neonate mouse offspring	1322	Male & female	Wildtype, Mice with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells, Transgenic mice with altered ENS, specifically modulated ENS activity	Specified below

Provide justifications for these choices

Species	Mice are used due to their anatomical, physiological, and genetic similarity to humans as well as their small size, ease of maintenance, short life cycle, and abundant genetic resources but particularly due to the established *in vitro* cell culture protocols
Origin	CD63 floxed from a licensed non-commercial breeder in USA, Sox10.CreERT2:R26tdTomato from a licensed non-commercial breeder within EU, Sox10.CreERT2/Wnt1.Cre:R26-GCaMP6f and Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD from licensed non-commercial breeders within Europe due to contributions/collaborations from other groups/institutes that share expertise, interests, genetically manipulated animal models. The remaining models are in house or will be obtained from a registered breeding facility.
Life stages	Adult – due to well-established *in vitro* protocols (Boesmans, Lasrado et al. 2015) (5.1 lid2e, 5.1 lid2h). Enteric neurospheres will be obtained from P1-P3 mouse of wildtype and transgenic mice (e.g. Wildtype, Wnt1.Cre:R26^{tdTomato}).
Number	In order to generate homogeneous and mixed ENS cultures (e.g., EGCs, neurons and progenitors), (and/or) adjacent normal epithelium, and intestinal organoids we need to isolate murine gastrointestinal tract. These cells will be used to investigate the role of ENS (ENS activity/ENS characteristics/vesicle secretion) in the gut, and their interplay with epithelial/tumor/immune cells (co-cultures with available cell lines). <u>ENS cultures:</u> Primary ENS cells will only grow for a maximum of 10 days and they do not divide, so we will need to isolate these cells every week. We prefer to isolate these cells from Sox10.CreERT2:R26tdTomato animals, as for most, but not necessarily all, experiments, we need fluorescently labelled ENS cells. In case we cannot generate decent/enough cultures using this line, we will (also) use wildtype animals. Given that with our optimised protocols we need the intestine of 2 mice to generate enough cells for one culturing procedure, will need 2 mice every week for 5 years, leading to a total of maximally **500** animals. Furthermore, other mouse lines with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells and transgenic mice with altered ENS or specifically modulated ENS will be used to describe specific cellular and molecular mechanisms for cell-to-cell communication. Therefore, we will need approximately 50 mice per genotype. We will use a maximum of 11 models/genotypes (genetically altered models described below and wildtype littermates for ENS altered models), so in total we will need **550** animals for this part. <u>Neurospheres:</u> Isolated neurospheres can be cultured for approximately 100 passages, so only a limited number of P1-P3 mouse neonates will be needed, as P1-P3 intestines contain a large number of proliferative ENS cells. However, we take into account that the isolation of neurospheres is a very new field, so protocols still have to be optimized using pilot experiments. From previous experiments performed by our collaborators, we know that a minimum of 8-10 P1-P3 intestines is required for each neurosphere isolation, so we need approximately 10x10 P1-P3 = 100 per genotype. For this aim we will use the Wnt1.Cre:R26^{tdTomato} line and (possibly) wildtype mice (see table 1), so **200** animals in total for this part. <u>Organoids:</u> Using previously adapted protocols from an experienced lab and Stem Cell technologies, we are able to generate abundant OGs from one intestinal section. OGs can be passaged/cultured for about 100 passages (12 mice per genotype for 5 years). For this aim we will be using NDRG4fl/fl:Wnt1.Cre, NSE-Noggin and Hand2fl/+:Wnt1.Cre mice with corresponding wildtypes (see table 1), so a total of **72** (12x6) animals. **Total: Max** 1322 mice
Gender	Both male and female mice will be used to minimise any gender-related variations in the experiment.

Genetic alterations	<table><tr><th>Mouse model</th><th>Potential examples</th><th>Readout</th><th>Number</th></tr><tr><td>Mice with fluorescently labelled ENS cells</td><td>Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}/Wnt1.Cre:R26-GCaMP6f</td><td>Creation of primary cell lines, neurospheres</td><td>500 + 100 200</td></tr><tr><td>Mice with fluorescently labelled extracellular vesicles from ENS cells</td><td>CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre</td><td>Creation of primary cell lines</td><td>50</td></tr><tr><td>Transgenic mice with altered ENS + corresponding WT</td><td>NDRG4$^{fl/fl}$:Wnt1.Cre, NSE-Noggin Hand2$^{fl/+}$:Wnt1.Cre</td><td>Creation of primary cell lines, organoids</td><td>300 72</td></tr><tr><td>Lines designed to specifically modulate ENS activity + corresponding WT</td><td>Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD</td><td>Creation of primary cell lines</td><td>100</td></tr></table> In the table above, the overall mouse models are described with specific examples of mouse lines that we want to use for this. However, as explained above, these lines are for now termed examples because we want to have the option of choosing a better model if those will be designed in the future. Each experimental model has its own purpose as different cell types/molecules will be labelled or present with different ENS-specific alterations. The specific reason and goal for the example lines that will be used to describe specific cellular and molecular mechanisms for cell behaviour and cell-to-cell communication are: • Sox10.CreERT2:R26tdTomato and Wnt1.Cre:R26tdTomato to label EGCs and/or enteric neurons • Sox10.CreERT2/Wnt1.Cre:R26-GCaMP6f for labelling and to investigate activity of EGCs and/or neurons • CD63f/+:Sox10.CreERT2:R26tdTomato or CD63f/f:Sox10.CreERT2:R26tdTomato to label, deplete and trace extracellular vesicles derived from EGCs • NDRG4fl/fl;Wnt1.Cre as enteric neuronal-specific knockdown of NDRG4 • NSE-Noggin and Hand2fl/+:Wnt1.Cre to study the effect of more respectively less enteric neurons • Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD to modulate ENS activity
Strain	(mixed) C57BL/6J (e.g. NDRG4fl/fl;Wnt1.Cre, Hand2fl/+:Wnt1.Cre, Sox10.CreERT2, R26-hM4Di-DREADD, R26-GCaMP6f), (mixed) 129S4SvJaeJ (e.g. Hand2fl/+:Wnt1.Cre), (mixed) FvB (e.g. NSE-Noggin). The strain of the wildtype mice will be matched to the corresponding transgenic mise used in the procedure.

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

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.

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

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

Genetically altered models:
Whereas Yoshimura et al described that adverse effects can occur in transgenic rats overexpressing human CD63-GFP (Yoshimura et al., Sci Rep., 2016), Men and colleagues have not reported CNS aberrations in mouse after inducible CD63 manipulation (Men et al., Nature Communications, 2019). This is in line with the observation of Yoshimura et al that the adverse effects of CD63-GFP overexpression can be circumvented by promoter-directed expression of the transgene in specific cell types of interest – similar as the procedure that will be applied here. Consequently, we do not expect any adverse effect when manipulating CD63 in the ENS. No adverse phenotype has been reported for the *Sox10.CreER*^{T2} mice (Laranjeira et al, JCI, 2011). Even though previous studies have described that the Hand2fl/+ x Wnt1.Cre mice might experience slowed gastrointestinal motility and constipation (Dáutreaux et al, Gastroenterology 2011), we now already have the mice for 2 years in our animal facility and we (researchers and CPV personnel) haven't observed this discomfort. Therefore, we don't expect to observe this harmful phenotype in this model.
For all the other lines described above, there are no data available describing adverse effects. However, based on the fact that the other models do not depict adverse effects, and that most of them merely represent reporter lines, we do not expect the appearance of deleterious phenotypes. Nevertheless, all animals will be closely monitored, and further actions (see pain relieve or humane endpoints) will be taken in case of change. To reduce stress for mothers of which we need neonates, we will set up the breeding in such way that we only have to use part of the litter for neonate experiments. The other pups will stay with the mother for normal length of time and will be used for breeding or experiments in the adult stage.

Administration of substances:
Based on experience and literature, we do not expect significant adverse effects from administration of transgene altering agents (e.g. tamoxifen) at any developmental stage. No more than transient discomfort caused by injection (max 2 times) at time of administration and no lasting harm are expected.
In case of effects in individual animals of particular scientific interest, the designated veterinarian will be requested for advice.

Explain why these effects may emerge.

As described above, we don't expect adverse effects from our genetically altered models. Administration of substances can induce mild, transient discomfort, because of the injection itself (injection site) or the substance.

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

Animals will be monitored daily by experienced people (Animal facility staff (caretakers/ biotechnicians) or responsible investigators). The mice will be adequately housed and working procedures will be adjusted. Experimental procedures will be done aseptically.

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.

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).
Mild (100%): - Administration of substances and labelling agents can cause short lasting mild pain and discomfort (transient) - Mice will be killed after the protocol, where after we collect tissues to isolate/prepare primary (3D)cell cultures.

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	If possible, we will perform the experiments using established *in vitro* cell lines. Only when these techniques are not optimal to investigate our research questions, or if they do not work, will we use the primary cell cultures to investigate our hypothesis. For example, no ENS cell lines are available to study our research questions, therefore primary cell cultures need to be used. We aim to validate our data using cell cultures derived from human intestinal tissue specimens. However, compared to the high murine cell yield and tissue availability, a limited number of human tissue samples is available and the cell yield is also narrow, thereby limiting the possibility to adequately and preferentially use human samples. Furthermore, human samples cannot be easily manipulated to have the same potential as our mouse lines.
Reduction	We will limit the number of animals by isolating different cell types from the intestinal tract of the same mouse and by using animals that come from breedings carried out for the procedures in appendix 2/3, but cannot be used there.
Refinement	We do not expect to culture cells from any animals that display harmful phenotypes. Our experimental *in vitro* approach will allow us to gather very specific insights prior to doing any *in vivo* procedures.

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.

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.
N/A
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.

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.
N/A

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.

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.

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

It is necessary to kill the animals to extract cells to start primary (3D) cell cultures.

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.

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

☒ No > Describe the method of killing.

CO_2 inhalation and cervical dislocation

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

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

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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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

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.

Using the animal lines (table 1), models and procedures specified in this appendix, we aim to address RQ2 of our PL and investigate/ unravel the interaction of ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal cells (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (e.g. microbiota) in health and colorectal cancer (CRC) *in vivo*. Hereby, we will explore the interaction between the ENS and CRC and the potential underlying mechanisms.
In addition, we will isolate tumors and adjacent normal epithelium, to generate intestinal (tumor) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact intra- and extracellular mechanism by which the ENS affects the epithelial and tumor cells. In case of success in acquisition and analysis of data using these models (e.g. differences in cancer induction (tumor number/size/growth) between genetic models) (go/no-go), we will also study the potential impact of an impaired ENS (genetically/chemically-induced alteration) on current treatments of CRC (e.g. radio- and chemotherapy). We will use wild-type mice and mice with a labelled or altered ENS (see table below) containing a non-diseased gut (control) and/or a diseased gut (colorectal cancer) (**Figure 1**).

Mouse lines:

Table 1: Mouse models and potential examples of mouse lines that can be used*:

Mouse model	Potential examples
Wild-type mice	

Mice with fluorescently labelled ENS cells	Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}/Wnt1.Cre:R26-GCaMP6f
Mice with fluorescently labelled extracellular vesicles from ENS cells	CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre
Transgenic mice with altered ENS	NDRG4$^{fl/fl}$Wnt1.Cre, (enteric neural specific Ndrg4 knockdown) NSE-Noggin (more enteric neurons) Hand2$^{fl/+}$:Wnt1.Cre (less enteric neurons)
Lines designed to specifically modulate ENS activity	Sox10.CreER^{T2}/Wnt1.Cre:R26-hM4Di-DREADD

* The mouse lines depicted within this table are at present the best models to investigate our research question. However, we would like to point out that better models might appear in the future (e.g. more efficient labelling of alteration of cells/molecules of interest). Consequently, the lines given here represent potential examples, by which means we will be able to change to better models in case these will be designed and/or become available. However, the number of models will remain the same.

Several primary outcomes **(see figure 1 – orange box)** will be analysed by using one, or a combination of the animal models described above (table 1)**:**

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)
- ENS function (e.g., ENS connectivity and network activity)
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)
- Development/progression of CRC (e.g., track tumour burden)
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)
- Microbiota analysis (e.g., composition and abundance, bacteria secretome)

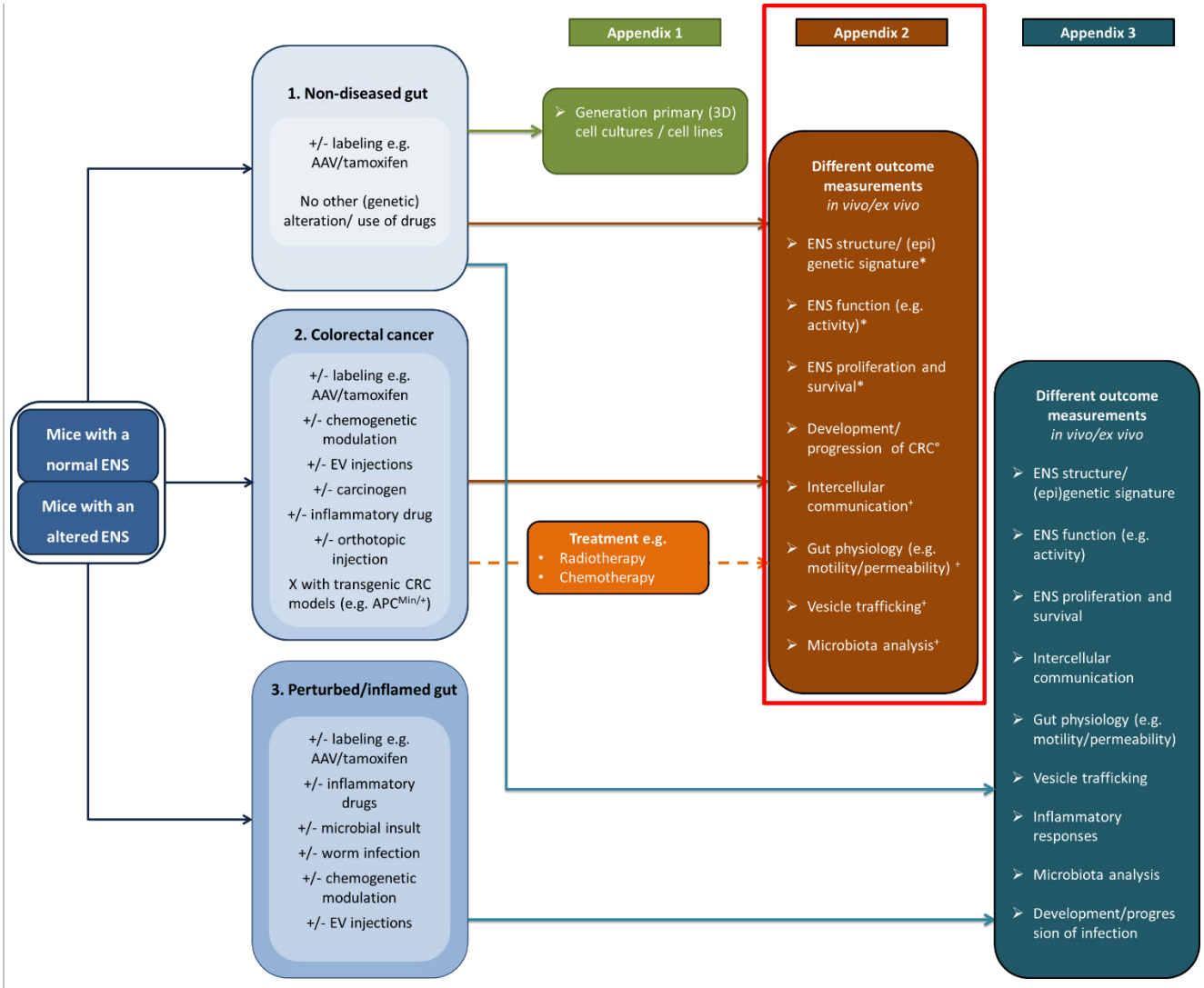

Figure 1. Outline depicting experimental models and outcome measures that will be assessed within each appendix. Red box delineates the purpose of this appendix.
With the experimental models and outcomes, we expect to further unravel mechanisms by which the ENS influences/responds to neighbouring environment in colorectal cancer by addressing the sub aims pointed below.

Aim 2: Investigate cellular and molecular mechanisms of interaction between ENS, intestinal (cancer) and other cells in a cancerous environment
Aim 2a: The role of EGCs in the development, progression, and treatment of CRC
Aim 2b: The role of enteric neurons in the development, progression, and treatment of CRC

This research will follow up on the research carried out under our previous project license 2017-026. Whereas animal lines, disease models and procedures partially overlap with PL2017-026, we have prevented as much overlap as possible. This is visualized in the table with all mouse numbers (word document – Total number of mice appendix 2) – where we omitted from the calculation of number of animals, the experiments/procedures that have already been performed. The data of these experiments demonstrate that the mouse lines and disease models are appropriate for studying the same outcome measures in other mouse lines and new outcome measures on both already in use and new mouse lines. The only overlap in animals that is present in both licenses is the therapy part as there is not enough time on the old project license to complete these experiments. This is due to the delay in establishing the correct mouse models and the Covid-19 pandemic.

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

To study how the ENS interacts with other cells, tissues and systems in health and CRC *in vivo* and *ex vivo*, we will take advantage of the well-established mouse lines described above (table 1). Animals can be subjected to CRC **($APC^{Min/+}$, AOM, AOM/DSS, and/or orthotopic CRC model),** (epi)genetic editing (**AAVx-carrying genetic modifiers)**, and/or chemogenetic modulation **(DREADDs)** as described in the details below and in their respective programs of work. The same lines can be further examined after treatment for cancer **(radio- and chemotherapies)**.

Animal procedures for specific mouse models mentioned in table 1:

1. **Genetic editing of the ENS using viral vector transduction (AAVx.transgene) –** AAVs will be used to deliver genetic material to the ENS of mice to induce genetic alterations (e.g., deliver fluorescent reporters and/or silence, knockdown or overexpress genes) by e.g. tail vein injection, to analyse the response of the intestinal and extra-intestinal tissues.

2. **Chemogenetic modulation using designer receptors exclusively activated by designer drugs (DREADDs) -** AAVx vectors will be injected (e.g. tail vein injection) to target designer receptors exclusively activated by designer drugs (DREADD) variants to modulate ENS activity (e.g., activate or inhibit EGCs/enteric neurons). ENS structure, function and intercellular communication as well as gut physiology can be subsequently analysed**.** Similarly, DREADD receptors will be targeted to ENS cells using Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD mice. Receptor activation will be achieved by providing clozapine-N-oxide (CNO) to the animals (via drinking water or ip injections).

Animal procedures for non-diseased or control vs diseased mice using the mouse models described in table 1:

1. **Control group** (e.g., untreated/sham mice and/or homozygous wildtype control with no expected deleterious phenotype)
    - For the mice with fluorescently labelled ENS cells and fluorescently labelled extracellular vesicles from ENS cells, this will be untreated/sham mice (so no cancer induction), but containing the labelled ENS cells or EVs.
    - For the transgenic mice with altered ENS and the lines designed to specifically modulate ENS activity, this will be (homozygous) wildtype controls that underwent the same procedures/colorectal cancer induction protocols as the transgenic mice.

2. **Colorectal Cancer (CRC) –** The following models will be used to trigger the onset of CRC.
    - *Genetic model:* $APC^{Min/+}$ mice spontaneously develop tumours predominantly in the small intestine within approximately 6 months. Based on previous observations with our NDRG4 mice, we expect the development of about 30 tumours in all models, resulting in mild discomfort. This model can be combined with the carcinogen AOM (injection) or the inflammatory drug DSS (in drinking water) (for more details, see the two model below) to induce tumours in the colon as well.
    - *Chemical carcinogen model*: Young adult mice (1-3 months) will be introduced to a well-established AOM carcinogen protocol (1 ip injection per week for 6 consecutive weeks). Tissues will be collected and analysed four months after the last AOM injection. Based on our previous observations by using this protocol, we expect the development of about 2-10 small colonic polyps in the models, causing mild discomfort.
    - *Chemical inflammatory/carcinogenic model:* Mice will be exposed to a combination of the inflammatory drug DSS (in drinking water) and the carcinogen AOM (ip injection), giving rise to colitis-associated cancer. Based on previous experience, we expect moderate discomfort and the appearance of about 10-40 colonic polyps, in the combined AOM/DSS model, but the development/growing of these polyps is faster than the AOM only model.
    - *Orthotopic CRC model:* Tumour cells (e.g. MC38 or CT26 cells) will be injected into the colon using a colonoscopy device when mice are under anaesthesia. A tumour will arise in every injection site (maximally three per mouse). Tumour metastasis and response to therapy (chemotherapy/radiotherapy) can be assessed in this model by CT imaging and evaluation after killing. Moderate to severe discomfort is expected.

      ***To evaluate the contribution of the ENS to the onset and progression of the disease, mice may also be killed prior to the appearance of polyps. However, CT scans can also be used for this purpose.**
      *** All CRC models used in this study do not have metastatic potential, so the mice will only develop tumours within the colon or small intestine, depending on the model used and the injection site.**

All four models have their specific characteristics and (dis)advantages. However, the chemical AOM (CRC model) and AOM/DSS (colitis-associated CRC model) model are the preferred models, because of the faster and more efficient CRC development and will therefore be used to study most of the outcome measures. Nevertheless, we will apply the genetic and orthotopic models to answer specific questions like labelled cell tracking (therapy experiments) and specific insights in human-specific CRC pathogenesis (when AOM or AOM/DSS models are not able to study certain outcome parameters with enough relevance for the patient). In addition, we will treat mice with CRC with either radiotherapy or chemotherapy, which can give us more insight into the mechanisms of treatment effects and the patients that would benefit from these treatments. Controls will receive sham therapy.

- Radiotherapy: Irradiation (non-lethal dose; therapeutic efficacy) onto the tumor tissue (via CT imaging): frequency: Max to be determined (range 5-10); Duration: max 30 min. Further details: Animals have to be food deprived before performing a CT scan (time depending on day-night rhythm, max 16h), so no fecal pellets remain in the colon during imaging. At different time points after CRC induction, mice will be anesthetized for micro-CT imaging. The target intestinal tumors will be delineated, where after a beam will be placed as such to cover the target and to ensure optimal sparing of organs at risk. Around the tumors, a 1-mm margin will be included to ensure also irradiation of microscopic disease spread. These procedures will take about 15 minutes in anesthetized animals. Upon obtaining the optimal treatment plan, the target will be irradiated. After the procedures, animals are allowed to recover. Moderate (to severe) discomfort is expected from these procedures.
- Chemotherapy: Administration of for example 5-FU + oxaliplatin (at present the most used treatment for CRC) or saline: i.p. injection, dose per injection to be determined, max 10 injections. Moderate (to severe) discomfort is expected from these procedures.
  Of Note: Pilot experiments for establishing appropriate doses of chemo- and radio-therapy are included and will be performed using our previous PL (AVD5.1 lid2h) to minimize the discomfort and will be a ***go/no-go moment****. By carrying out the pilot experiments, we will try to make sure that we are able to find the most efficient dose and minimize the discomfort to moderate. If the pilot studies do not give rise to changes in tumor growth or if the discomfort on the animals is too high and cannot be minimized to moderate on average, we will not continue with the therapy studies included in this PL. However, given that these pilot experiments have not yet been performed, we have now taken into account the worst-case scenario, wherein animals may experience severe discomfort.*

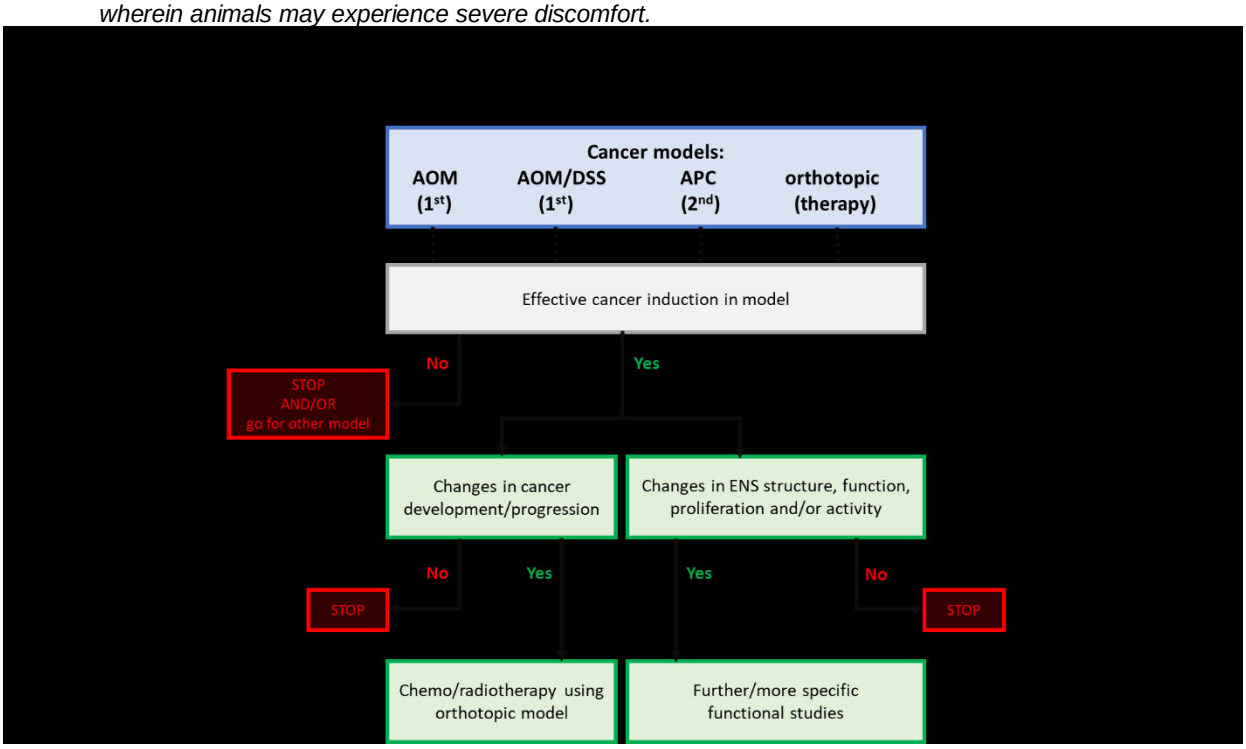

Figure 2. *Flowchart depicting the strategy and decision moments of the models (described above)/outcome measures (described below) in appendix 2.*

OUTCOME MEASUREMENTS THAT WILL BE ANALYSED:

As mentioned above, we will perform *in vivo* and/or *ex vivo* (post-mortem) analysis of the following outcome measures (the experimental methodologies that will be used are included as well). **Rationale: these outcome measures are included because they are ideally suited to reflect on the status and composition of the ENS, its modes of communication with, and effect on, surrounding tissues (e.g. tumour cells) and cellular systems (e.g. microbiota), and role in gut homeostasis (please also see background of Project Proposal).**

NB – Many of the outcome parameters can be analysed by using one group of animals (e.g., ENS structure, intercellular communication, gut physiology and microbiota analysis). Support on the group sizes and total number of animals can be found at section B. The subdivision of number of animals per procedure/model and the total number is depicted in detail within the following Word document: Total number of mice; table – appendix 2 – wherein we refer to the similar enumeration as used below in our explanation. As explained before, the mouse models in these tables are used as examples but can be substituted if better models become available.

- ***ENS structure/ (epi) genetic signature & ENS function & ENS proliferation and survival***

  Distinct imaging techniques will be used to study these parameters on ENS.

- We will examine the influence of CRC on the ENS composition, architecture, morphology and activity (**ex vivo)**:

a) ***Fate mapping experiments*** - we will use *Sox10.CreER^{T2}:R26td^{Tomato}*. Tamoxifen (i.p.) will be injected at/before the time of initiation of the CRC protocol to label Sox10 expressing EGCs with tdTomato reporter. **– 12 animals per group.**

b) ***Calcium imaging of ENS activity*** - We will examine the ENS activity *ex vivo* in different ways: i) [Ca2+]i-Fluo-4 imaging: Live recording of ENS activity will be performed using Fluo-4 Ca2+ -imaging in preparations of whole-mount mouse gut. ii) For the other Ca2+ imaging experiments we will use Sox10-CreERT2::R26-GCaMP6f mice (EGCs, tamoxifen injection in adult animals) and Wnt1.Cre:: R26-GCaMP6f mice (all enteric neurons and glia). **– 12 animals per group**

- We will examine the influence of CRC on the ENS regarding cell proliferation and survival

c) **For cell proliferation and survival:** Analysis of ENS proliferation at different stages during CRC development/progression. Animals will be given a thymidine analogue (e.g. BrdU, edU) to label cycling cells via i.p. injections and/or drinking water. *In vivo/ex vivo* labelling assays will be used to detect cell death, senescence, DNA damage. We will perform this experiment only if we observe any phenotype in organisation, composition and function of the ENS. **– 12 animals per group**

- We will investigate the molecular signature of ENS cells and/or extracellular components in gut homeostasis and CRC. We will use FACS to isolate ENS cells for qRT-PCR, bulk or single-cell RNA Sequencing

d) **Tissue isolation, digestion and FACS**
Intestinal preparations will be dissociated into single cell suspensions and subjected to FACS for isolation of ENS cells and/or other components and analysis by qRT-PCR, bulk and single cell RNA Sequencing. **- 9 animals per group**

- ***Development/progression of CRC***

  We will closely follow the onset and progression of tumours using the following techniques:

a) High resolution endoscopic monitoring - to analyse the effect of the treatment on tumour burden at different time points *in vivo*. **No culling, so performed in the mice that will be used at c.**

b) CT scans: This procedure will take about 15 minutes and will be done under anesthesia to prevent repositioning the animal. Contrast will be enhanced to visualize tumors using a protocol established in our lab and consists of an i.p. injection and a rectal injection of contrast agents. **No culling, so performed in the mice that will be used at c.**

c) All other outcomes will be analysed ex vivo (tumour number, size, histology, RNA expression, protein levels, cell isolation, neuron/EGC tracing, blood sampling etc.) **12 animals per group** to study histology and **6 animals per group** to study RNA/protein/cells.

d) This outcome measure will also be examined for the animals subjected to chemo- or radiotherapy. However, we need pilot studies with a smaller number of mice to first optimize the procedure before starting the experiment (go/no-go moment).

- ***Intercellular communication – 6 and 9 animals per group***

  We will investigate cell-to-cell communication between ENS cells and other intestinal and extra-intestinal systems (e.g. immune system, tumour cells, brain) by immunohistochemistry, qRT-PCR, FACS, western blotting, ELISA, etc. Mice will be euthanised and intestinal and extra-intestinal tissues will be collected for subsequent analysis. We will also isolate tumors and adjacent normal epithelium, to generate intestinal (tumor) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact mechanism by which the ENS affects the epithelial and tumor cells.

  NB: The tissue collection will be performed separately only when it cannot be achieved together with other experiments, in order to minimise unnecessary culling and animal use.

- ***Gut physiology (intestinal motility & gut permeability)***

- To study intestinal motility ***in vivo*** we will implement different methods:

a) *Total intestinal transit time*: The protocol for the total gastrointestinal transit time will be performed as previously described[5]. Mice will be individually placed into cages devoid of bedding and fasted for an hour. Next, a dye will be administrated by oral gavage, and the animals returned to their individual cages. The total intestinal transit time will be calculated by the time from gavage until the extrusion of the first coloured pellet. A maximum period of observation will be 5 hours in all experiments and mice that fail to expel the red pellet within this time will be quantified as ending point at 5 hours. ***NB: No culling is needed, and mice can be used in other read-out parameters. The dye is cleared from the gastrointestinal tract 2 days after administration. Faeces will be collected and analysed for bacterial composition and stool quality.**

b) *Small intestinal transit:* As described for the total transit time, mice will be given dye via oral gavage. Mice will be euthanised, followed by removal of the small intestine. The total length of the small intestine and the distance that the dye has travelled will be measured to determine the small intestinal transit length. ***Faeces will be collected and analysed for bacterial composition and stool quality. Tissues will be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain).**

c) Colonic propulsion: Colonic propulsion will be evaluated by first lightly anesthetizing the animals with isoflurane. A small diameter glass bead will be inserted into the rectum, and the expulsion time of the glass bead will be recorded. The assay will be repeated twice every 100 min for a total of 3 bead insertions. ***NB: No culling is needed, and mice can be used in other read-outs.**

NB – experiments in a and c can be performed using the same group of animals – 12 animals per group.

- **In case of a phenotype in a, b and/or c -** To study the intestinal contractility and motility ***ex vivo*** using live video recording and spatiotemporal analyses of ENS-dependent motility. Experiments can be performed using tissue derived from animals used in b.

d) Segments from distinct parts of the gut of young adult mice will be removed as previously described. Segments will be carefully isolated, luminal contents emptied and placed loosely pinned onto an organ bath chamber continuously infused with Krebs solution and constantly supplied with 95% O2 and 5% CO2 at 37°C and the neurogenic intestinal motility recorded.

- To study gut permeability ***in vivo***:

e) *Measuring levels of plasma FITC:* - Intestinal permeability will be determined by measuring levels of plasma FITC after administration via oral gavage of FITC-conjugated dextran in PBS. Blood will be obtained after administration, and the concentration of fluorescein will be determined by spectrophotofluorometry. Mice will be euthanized during this experiment. ***Faeces can be collected and analysed for bacterial composition and stool quality. Tissues can be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain). – 12 animals per group**

- ***Vesicle trafficking analysis***

- Distinct imaging techniques will be used to study ENS vesicle trafficking **– 12 animals per group**:

e) FM1-43 imaging ***(in vitro)***: We will investigate how synaptic signalling and synaptic vesicle turnover (FM1-43) differ in ENS cultures derived from the different mouse models by using FM1-43 imaging and after distinct stimuli (chemical, electrical).
f) NTA analysis ***(in vitro*** and ***ex vivo)*** - To analyse pattern parameters of EVs: intensity fluctuations, surface geometry and shape of the particles as well as particle concentration to distinguish sub-populations of vesicles.
g) Manipulation of EVs by viral vector targeting in the ENS ***(in vivo)*** - AAVx.XFP carrying genetic modifiers to silence (shmiRNA) or overexpress molecules of interest, will be injected (i.v.) in our animal models to evaluate the role of EVs in the maintenance, organisation and function of the ENS and in CRC. No side effects are expected with AAV injections (mild discomfort). ***NB – Most of these experiments described above can be performed using the same group of animals as they are likely to cause transient and mild pain and discomfort.**

- ***Microbiota analysis***

Metagenomic DNA extraction from faecal, and tissue samples and molecular profiling/16S rRNA sequencing will be carried out to explore differences in the microbial community structure between experimental groups. We will examine the differences in microbial richness (e.g., *Chao1*) and diversity (e.g., *Shannon*). ***NB: The tissue collection will be achieved together with other experiments, in order to minimise unnecessary culling and animal use, so no extra animals will be used for this outcome measure.**

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

For the quantitative experiments, design has been based on ARRIVE guidelines and sample sizes have been set using power analysis https://www.statstodo.com/SSizCorr_Pgm.php. to determine the number of mice needed for each experiment, generally considering a difference between groups of at least 20%, power of 80% and significance level of 5%. For qualitative experiments, we will use the minimum number of mice to provide an accurate description based on previous publications and on our own experience, also from our previous PV.

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

1	Mouse	In-house breeding	(Young) adults	2118	Male and female	Wildtype, Mice with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells, Transgenic mice with altered ENS, specifically modified ENS activity	

Provide justifications for these choices	
Species	Mice due to their anatomical, physiological, and genetic similarity to humans as well as their small size, ease of maintenance, short life cycle, and abundant genetic resources.
Origin	Sox10.CreERT2:R26tdTomato from a licensed non-commercial breeder in UK (but currently in house), Sox10.CreERT2:R26-GCaMP6f and *Wnt1.Cre:R26-hM4Di-DREADD* from licensed non-commercial breeders within Europe, NDRG4$^{fl/fl}$;Wnt1.Cre, NSE-Noggn and Hand2$^{fl/+}$;Wnt1.Cre are all in house, CD63 floxed from a licensed non-commercial breeder in USA
Life stages	Young adults (1-10 months) is most apt for optimal results
Number	Based on the literature, on our own and other collaborators experiences, we estimate that we will need a maximum of 12 mice per group for most outcome measures (high variability/spread expected because of the measurement method (for example, gut transit times vary a lot depending also on whether mice are very active or passive at the time of the experiment; tumor development/progression is very variable and tumors are divided in multiple grading groups, so you need multiple tumors per mouse for analysis (5.1 Ild2e, 5. et al 2017; 5.1 Ild2e, 5. et al 2021)). For FACS experiments, we will need max 9 mice per group (less tumor material needed, so less drop-out expected and spread is usually low (unpublished data)) and 6 mice for RNA experiments (less tumor material needed, so low drop-out expected and also low spread expected; RNA can partly also be obtained together with other outcome parameters (unpublished data; 5.1 Ild2e, 5. et al 2017; 5.1 Ild2e, 5. et al 2021)). In total, this will be maximally 2118 animals. For a more detailed overview of how this number is built up, we refer to the following Word document: Total number of mice appendix 2.
Gender	Both male and female mice will be used to minimise any gender-related variations in the experiment.
Genetic alterations	Mice with fluorescently labelled ENS (e.g. Sox10.CreERT2:R26tdTomato, Wnt1.Cre:R26tdTomato, Sox10.CreERT2:R26-GCaMP6f) will be used to study the interaction between the ENS and CRC. Specific ENS cells will be labelled and can thereby be easily followed in these models. Furthermore, activity and specific ENS cells can be studied. This will give us more insight into the effect of CRC on specific ENS cells and their activity and therefore we will get a better idea about possible interactions between these cell types. Mice with fluorescently labelled extracellular vesicles from ENS cells (e.g. CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre) can be used to study the involvement of extracellular vesicles in the interaction between the ENS and CRC. Transgenic mice with altered ENS (e.g., NDRG4fl/fl;Wnt1.Cre - Enteric neuronal-specific knockdown of NDRG4, Hand2f/+;Wnt1.Cre mice - more enteric neurons, and NSE-noggin - less enteric neurons) will be used to study whether neuron density or knockdown of NDRG4 (biomarker for CRC and specifically expressed in enteric neurons) affect CRC development/progression and which mechanisms play a role in this effect. Lines designed to specifically modulate ENS activity (e.g. Sox10.CreER^{T2}/Wnt1.Cre:R26-hM4Di-DREADD) will be used to study the effect of activation or inhibition of a specific subtype of ENS cells to the development/progression of CRC. This will give us insight into the role of ENS cells in CRC.

	Altogether, this may lead to possible pathways and/or targets that could be used for treatment in the future.
Strain	(mixed) C57BL/6J (e.g. NDRG4fl/fl;Wnt1.Cre, Hand2fl/+:Wnt1.Cre, Sox10.CreERT2, R26-hM4Di-DREADD, R26-GCaMP6f), (mixed) 129S4SvJaeJ (e.g. Hand2fl/+:Wnt1.Cre), (mixed) FvB (e.g. NSE-Noggin). The strain of the wildtype mice will be matched to the corresponding transgenic mice used in the procedure.

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

If mice have to undergo CT imaging, they need to be food deprived before performing the CT scan, so that no fecal pellets are present in the colon during imaging. Food will be given immediately when animals have undergone their CT scan.

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.

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

During the development of CRC or during CRC treatment animals might experience pain (see below). All possibilities to reduce pain, fear or suffering will be used. These might include use of appropriate analgesia (e.g. opiates (not in experiments of gut motility) or NSAIDs (not in experiments involving inflammation)) and anaesthesia (e.g. isoflurane) that do not affect gut motility, lead to gut abnormalities or affect the intestinal inflammatory response. However, use of analgesia is not expected based on experience with our past experiments, but pain will always be scored and evaluated to ensure proper management of the animals (if we cannot apply analgesia and animals experience discomfort, HEPs will be applied when necessary). Follow-up of the animals will be done regularly, at least daily, to ensure rapid notifications of signs of discomfort. Obviously, after experimental procedures e.g. injections/gavage, the animals will be followed up more frequently.

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

- *In vivo* experiments can cause distress or discomfort to the animals. Therefore, all animals will be frequently monitored for any signs of pain and distress. Changes to normal and provoked behaviour, movement, physical signs such as posture, respiration, skin and coat changes, inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist. Any mice exhibiting any deviation from the normal health and behaviour will be further monitored and treated accordingly.
- Genetically altered mice used in this appendix are not expected to show deleterious phenotypes.
- CRC mouse lines ($APC^{Min/+}$) and models (AOM and AOM/DSS) can develop side effects, such as pain, weight loss, diarrhoea/constipation and anal bleedings (no ulcers with perforation are expected).
- Radio- and Chemotherapies can induce necrosis, fibrosis and inflammation in irradiated tissues, and skin conditions may appear leading to loss of coat pigmentation depending on drug concentration. Furthermore, chemotherapy can induce side effects such as illness/sickness/nausea and reduction of appetite.

Explain why these effects may emerge.

- Most of the side effects mentioned above are unlikely in our non-diseased mice models (unless provoked), but can occur due to inflammation and/or progression of disease, development of polyps and alteration in intestinal microbiota.
- Radio- and chemotherapy can cause toxicity in normal/healthy tissues due to production of reactive oxygen/nitrogen species (ROS). This may result in immediate cell death, inflammation, tissue fibrosis and DNA damage in neighbouring tissues.

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

Animals will be frequently monitored by experienced staff and researchers. Our mouse colonies will be housed and maintained according to the basic guidelines for animal welfare. Working protocols will be adequately adjusted to benefit not only the research but also animal welfare. Experimental procedures/routes of administration, techniques that cause the least pain, suffering, distress and the shortest lasting harm will be preferred. Experimental procedures will be performed by using aseptic/sterile techniques, and drugs such as analgesics, anaesthetics and antibiotics will be administered whenever necessary. Examples of measures that we will use to prevent or minimise harm are minimizing radiotherapy and chemotherapy dosages and times to what is minimally required, extra hydration in case of diarrhea and boosting gels in case of high weight loss. Measures will be taken accordingly in the case any unexpected adverse effects might appear and animals will be immediately euthanized and experiments stopped in the case of unexpected severe signs of pain and discomfort.

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.

- All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist.
- Any animal will be immediately euthanised if it exhibits signs of suffering that is not transient or treatable, and compromises its normal behaviour.
- Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe discomfort and >20% for severe discomfort) that persist for 24 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision.
- Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled.
- Chemical CRC models: animals undergo euthanasia about 100 days after the beginning of the procedure. Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 2), “Wasting Syndrome” (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), or opportunist infections, the animals will be immediately euthanised.
- Radio- and Chemotherapies: adverse effects such as inflammation and skin conditions such as loss of coat pigmentation may appear. Animals can undergo moderate to severe adverse effects and will be culled if signs of severe discomfort persist such as facial expression score of 3, “Wasting Syndrome” (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), coat/skin condition 3 for at least 3 consecutive days, chronic diarrhea or opportunist infections.

Indicate the likely incidence.

For the CRC models, and in the wildtype x CRC mice that underwent chemo or radiotherapy (±10-15% incidence). The animals will be well monitored by experienced people, so that we are able to rapidly detect unforeseen adverse effects in early stages, whereby we aim to avoid reaching the humane endpoints.

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).

Appendix 2	Non-diseased gut (I)	APC$^{Min/+}$ mouse model (II)	AOM model (III)	AOM/DSS model (IV)
Discomfort model itself → Discomfort procedures per outcome measure ↓	**No discomfort**	**Mild**	**Mild**	**Moderate**
ENS structure /function /proliferation - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Gut physiology - Administration of substances: **mild** - Transit/motility assays: **mild** - Killing: **mild**	Mild	Moderate	Moderate	Moderate
Vesicle trafficking - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Microbiota analysis - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Intercellular communication - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Development/progression of CRC - Administration of substances: **mild** - CT scans: **moderate** - Killing: **mild**	Moderate	Moderate	Moderate	Severe

Appendix 2: Treatment	AOM model (III)	AOM/DSS model (IV)	Orthotopic model (V)
Discomfort model + treatment (chemo/radiotherapy)→ Discomfort procedures per outcome measure ↓	**Moderate**	**Moderate**	**Moderate**
Development/progression of CRC - Administration of substances: **mild** - CT scans: **moderate** - Killing: **mild**	Moderate	Severe	Severe

Figure 3: *Cumulative discomfort for disease models or treatment models (columns) combined with outcome measurements and corresponding procedures (rows).* The different mouse lines itself (described in Table 1) are not expected to have any harmful phenotype or display signs of discomfort.

Approximate percentages expected cumulative discomfort based on animal numbers per outcome measure and disease model:
Mild: 50%
Moderate: 35%
Severe: 15% (max percentage, including the therapy studies that are subjected to a go/no-go moment)

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	To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models to study CRC. In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied *in vivo*. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established *in vitro* culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis *in vivo*. We are also taking advantage of recently established collaborations (e.g. in the gastroenterology clinic (5.1 lid2h to obtain human intestinal specimens and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study cancer development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Reduction	Before embarking on procedures in animal research, we will collect evidence form *in vitro/in silico* procedures if possible to determine whether a candidate gene or molecule also provide insights on *in vivo* pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This can also be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals (mostly included in the previous PL (AVD5.1 lid2h), so not included in the current strategy). This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.
Refinement	Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no phenotype and/or useful results, experiments that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and/or well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached.

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.

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.

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.

N/A

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.

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.

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

Ex vivo (post-mortem) and *in vitro* experiments will be performed in tissue and cells collected from our experimental groups to further analyse the biology of the nervous system.

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.

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

☒ No > Describe the method of killing.

CO_2 inhalation and cervical dislocation

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

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

Appendix 2							
Non-diseased gut (I)	***Sox10CreERT2/ Wnt1Cre-TdTomato (Ctrl AOM) (i)***	***Sox10CreERT2/Wnt1Cre-TdTomato (Ctrl AOM/DSS) (ii)***	***NDRG4$^{fl/fl}$ Wnt1^{Cre2} (iii)***	***NDRG4$^{fl/fl}$ (iv)***	***APC$^{+/+}$ Sox10CreERT2-TdTomato (v)***		
ENS structure /function /proliferation 1. Number/morphology (a) 2. Activity (b) 3. Proliferation/Survival (c) 4. FACS (d)	#/measure →12 →9	#/measure →12 →12 →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →12 →12 →9		**153**
Gut physiology 5. whole-gut + colonic (a, c) 6. small intestinal transit + ex vivo motility (b, d) 7. permeability (e)	#/measure →12 →12 →12	#/measure →12 →12 →12	#/measure	#/measure	#/measure →12 →12 →12		**108**
Vesicle trafficking 8. Imaging (a, b, c, d)	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12		**60**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis		**0**
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →9	#/measure →6 →9	#/measure →9	#/measure →9	#/measure →6 →9		**57**
Development/progression of CRC 11. Histology (c) 12. RNA/protein/cell isolation (c)	#/measure	#/measure →12 →6	#/measure	#/measure	#/measure →12 →6		**36**
							414

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h

Appendix 2								
***$APC^{Min/+}$* mouse model (II)**	***Sox10CreERT2 -TdTomato (v)***	***$NDRG4^{fl/fl}$ – $Wnt1^{Cre2}$ (iii)***	***$NDRG4^{fl/fl}$ (iv)***	***$Hand2^{fl/+}$ $Wnt1^{Cre2}$ (vi)***	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***	
ENS structure /function /proliferation 1. Number/morphology (a) 2. Activity (b) 3. Proliferation/Survival (c) 4. FACS (d)	#/measure →12 →12 →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	**171**
Gut physiology 5. whole-gut + colonic (a, c) 6. small intestinal transit + ex vivo motility (b, d) 7. permeability (e)	#/measure →12 →12 →12	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	**36**
Vesicle trafficking 8. Imaging (a, b, c, d)	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	**84**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	0
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →6 →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	**69**
Development/progression of CRC 11. Histology (c) 12. RNA/protein/cell isolation (c)	#/measure →12 →6	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	**18**
								378

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h .

Appendix 2									
***AOM* model (III)**	***Sox10CreERT2 /Wnt1Cre-TdTomato (i)***	***NDRG4$^{fl/fl}$ Wnt1^{Cre2} (iii)***	***NDRG4$^{fl/fli}$(v)***	***Hand2$^{fl/+}$ Wnt1^{Cre2} (vi)***	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***	***Sox10CreERT2:R26-hM4Di-DREADD or GCaMP6f (x)***	
ENS structure /function /proliferation 1. Number/morphology **(a)** 2. Activity **(b)** 3. Proliferation/Survival **(c)** 4. FACS **(d)**	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →12 →12 →9	**183**
Gut physiology 5. whole-gut + colonic **(a, c)** 6. small intestinal transit + ex vivo motility **(b, d)** 7. permeability **(e)**	#/measure →12 →12 →12	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →12 →12	**72**
Vesicle trafficking 8. Imaging **(a, b, c, d)**	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	**96**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	0
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →6 →9	**78**
Development/progression of CRC 11. Histology **(c)** 12. RNA/protein/cell isolation **(c)**	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →6	**18**
									447

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h .

Appendix 2									
***AOM/DSS* model (IV)**	***Sox10CreERT2/Wnt1Cre - TdTomato (ii)***	***NDRG4$^{fl/fl}$ Wnt1^{Cre2} (iii)***	***NDRG4$^{fl/fl}$ (iv)***	***Hand2$^{fl/+}$ Wnt1^{Cre2} (vi)***	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***	***Sox10CreERT2:R26-hM4Di-DREADD or GCaMP6f (x)***	
ENS structure /function /proliferation 1. Number/morphology (a) 2. Activity (b) 3. Proliferation/Survival (c) 4. FACS (d)	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →9	#/measure →12 →12 →12 →9	**183**
Gut physiology 5. whole-gut + colonic (a, c) 6. small intestinal transit + ex vivo motility (b, d) 7. permeability (e)	#/measure →12 →12 →12	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →12 →12	**72**
Vesicle trafficking 8. Imaging (a, b, c, d)	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	#/measure →12	**96**
Microbiota analysis Faecal + tissue samples	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	Combined with other analysis	0
Intercellular communication 9. RNA/ protein 10. FACS	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →9	#/measure →6 →9	**78**
Development/progression of CRC 11. Histology (c) 12. RNA/protein/cell isolation (c)	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure	#/measure →12 →6	**18**
									447

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h

***CRC treatment* models (V)**								
Chemotherapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+ AOM or***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***orthotopic***	→6	→6	→6	→6			**72**
11. Histology	***+ AOM/DSS***	→12	→12	→12	→12			
12. RNA/protein/cell isolation		→6	→6	→6	→6			**72**
Control chemotherapy – sham therapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+ AOM or***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***orthotopic***	→6	→6	→6	→6			**72**
11. Histology		→12	→12	→12	→12			
12. RNA/protein/cell isolation	***+ AOM/DSS***	→6	→6	→6	→6			**72**
								288

Radiotherapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***Orthotopic***	→6	→6	→6	→6			**72**
Control Radiotherapy – sham therapy								
		Hand2$^{fl/+}$ Wnt1^{Cre2}(vi)	***WT (vii)***	***NSE- noggin (viii)***	***WT (ix)***			
Development/progression of CRC		#/measure	#/measure	#/measure	#/measure			
11. Histology	***+***	→12	→12	→12	→12			
12. RNA/protein/cell isolation	***Orthotopic***	→6	→6	→6	→6			**72**
								144

TOTAL appendix 2 = 414 + 378 + 447 + 447 + 288 + 144 =	**2118**

Of note: for some of the models we have not put any numbers in the table, because these experiments have already been/will be performed under our previous PL: AVD5.1 lid2h .

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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: 3 Type of animal procedure: Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

In this appendix we aim to investigate and unravel the interaction between adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) and other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota) in health and/or upon local and systemic challenges (RQ3 of the PL).

Mouse lines:

Table 1: Mouse models and potential examples of mouse lines that can be used*:

Mouse model	Potential examples
Wild-type mice	
Mice with fluorescently labelled ENS cells	Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}:R26-GCaMP6f
Mice with fluorescently labelled extracellular vesicles from ENS cells	**CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre
Lines designed to specifically modulate ENS activity	Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD

* The mouse lines depicted within this table are at present the best models to investigate our research question. However, we would like to point out that better models might appear in the future (e.g. more efficient labelling of alteration of cells/molecules

of interest). Consequently, the lines given here represent potential examples, by which means we will be able to change to better models in case these will be designed and/or become available. However, the number of models will remain the same.

Several primary outcomes **(see figure 1 – dark blue box)** will be analysed by using one or a combination of the animal models described above**:**

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)
- ENS function (e.g., ENS connectivity and network activity)
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)
- Inflammatory processes (e.g., immune cell phenotype)
- Microbiota analysis (e.g., composition and abundance, bacteria secretome)
- Development/progression of infection (e.g., track parasitic infection and worm burden, numbers and eggs)

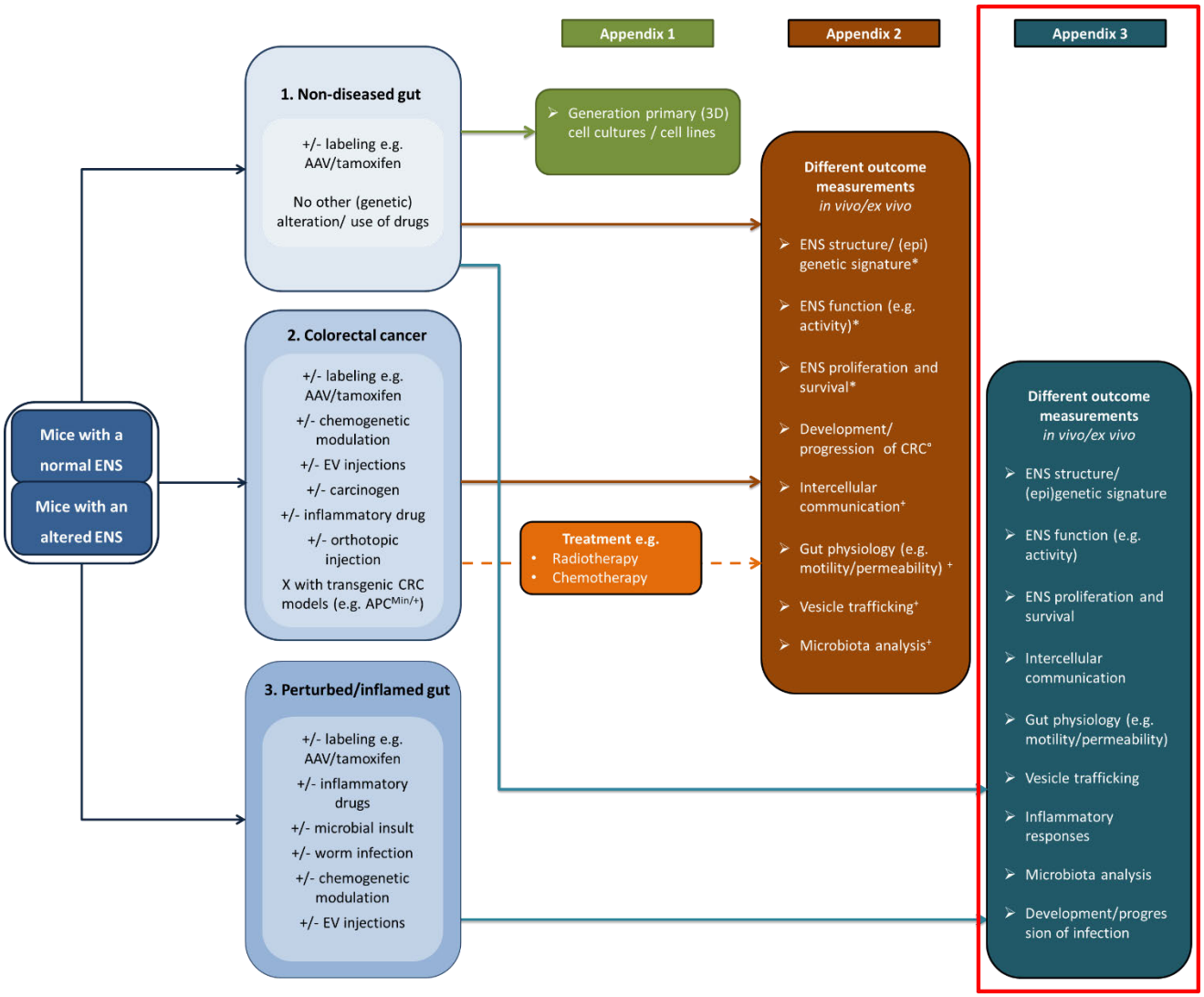

Figure 1. Outline depicting experimental models and outcome measures that will be assessed within each appendix. Red box delineates the purpose of this appendix.

With the presented experimental models and outcomes, we expect to further unravel mechanisms by which the ENS influences/responds to neighbouring environment by addressing the sub aims pointed below.

Aim 3: Investigate the mechanisms of cell-to-cell communication in homeostasis and diseases that affect the gastrointestinal tract (e.g., **worm infection**, (and/or) microbiota alteration, (and/or) inflammation).

Aim 3a: The role of ENS-derived EVs for cell-to-cell communication in health and disease.
Aim 3b: The role of ENS-derived miRNAs

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

To study how the ENS interacts with other cells/tissues and systems in health and disease *in vivo* and *ex vivo*, we will take advantage of the well-established mouse lines described above. Animals can be subjected to worm infection **Heligmosomoides polygyrus (*H. poly*),** (epi)genetic editing (**AAVx-carrying genetic modifiers)**, intestinal inflammation **(DSS/BAC),** microbiota alterations **(depletion/antibiotics),** chemogenetic modulation **(DREADDs)** as described in detail below and in their respective programs of work. The same lines can be further examined by rescue experiments to re-establish the intestinal microbiota composition **(reintroduction of microbes by faecal transplantation)**, rescue of specific phenotype by using advanced technology to deliver molecules **(AAVx.transgene).**

Animal procedures for specific mouse models mentioned in table 1:

- **Genetic editing of the ENS using viral vector transduction (AAVx.transgene) –** AAVs will be used to deliver genetic material to the ENS of mice to induce genetic alterations (e.g., deliver fluorescent reporters and/or silence, knockdown or overexpress genes) by e.g. tail vein injection, to analyse the response of the intestinal and extra-intestinal tissues. To our knowledge, literature doesn't describe a harmful phenotype induced by these injections, so there will be no more than mild discomfort.

- **Chemogenetic modulation using designer receptors exclusively activated by designer drugs (DREADDs) -** AAVx vectors will be injected (e.g. tail vein injection) to target designer receptors exclusively activated by designer drugs (DREADD) variants to modulate ENS activity (e.g., activate or inhibit EGCs/enteric neurons) (max. mild phenotype). ENS structure, function and intercellular communication as well as gut physiology can be subsequently analysed**.** Similarly, DREADD receptors will be targeted to ENS cells using Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD mice. Receptor activation will be achieved by providing clozapine-N-oxide (CNO) to the animals (via drinking water or i.p. injections). We will perform shRNA knockdown of ENS genes using AAVx vector technology to modulate ENS cells. To target critical stages of ENS network formation, these interventions can be performed during late prenatal, early postnatal stages of ENS development, or adult mice of either sex.

Animal procedures for non-diseased or control vs diseased mice using the mouse models described in table 1:

1) **Control group** (e.g., untreated/sham mice and/or homozygous wildtype control with no expected deleterious phenotype)
- For the mice with fluorescently labelled ENS cells, fluorescently labelled extracellular vesicles from ENS cells and the lines designed to specifically modulate ENS activity, this will be untreated/sham mice but containing the labelled/activity modulated ENS cells or EVs.

2) **Perturbed/Inflamed gut –** Various challenges will be used to affect gastrointestinal homeostasis and trigger an ENS response (see figure 1 for breakdown of go/no go decision moments):
- Worm Infection – *H. poly* are mouse pathogens. While wild-type mice are able to clear the worm without exhibiting significant clinical problems, mice carrying ENS-specific mutations may be more susceptible to pathology. Infected mice are expected to have mild discomfort. Mice will be infected with *H. poly* third stage (L3) larvae by oral gavage, which were obtained from faecal cultures of H. poly-infected mice from a collaborating lab (Johnston et al, J Vis Exp, 2015). The number of adult worms in the intestinal wall at day 7 of infection, the number of adult worms in the intestinal lumen and the number of macroscopically visible granulomas will be enumerated by manual counting (5.1 lid2e, 5.1 lid2h
- Genetic editing: (Go/No-go) If we find specific targets of interest (e.g. microRNAs) that are dysregulated in the ENS in the worm infection model we will use advanced technology to deliver molecules (AAVx.transgene) – AAVs will be used to deliver genetic material to the ENS of mice to induce genetic alterations (e.g., silence, knockdown or overexpress genes) by e.g. tail vein injection, and thus, we can then analyse the response of the intestinal and extra-intestinal tissues.
- Intestinal Inflammation (systemic and focal): (Go/No-go) These models will be used if we find an immune related phenotype (e.g. Immune pathway activations, immune cell perturbances) in the worm infection model. In this case, we will use well-known chemicals that trigger systemic (dextran sodium sulfate – DSS; in drinking water) and focal

(benzalkonium chloride – BAC) intestinal inflammation. To induce acute or chronic inflammation, with different degrees of severity, animals will be subjected to DSS treatment (varying time frame and concentrations).
For BAC – BAC will be surgically applied onto the serosal surface of the intestine by laparotomy (distal ileum or proximal colon). Mice will be closely monitored, and tissues harvested after surgery. *To evaluate the contribution of the ENS to the onset and progression of the disease, mice will also be collected prior to the development of inflammation.

- Alteration in intestinal microbiota (Antibiotic treatment and microbiome reconstitution): (Go/No-go) These models will be used if we find alterations in the intestinal microbiota (e.g. disruptions in the communication between ENS and intestinal microbiota) in the worm infection model. In this case, young adult mice or pregnant females will be subjected to single or a cocktail of broad-spectrum antibiotics in their drinking water (chase) or oral gavage (pulse). The antibiotic cocktail consists of ampicillin, metronidazole, vancomycin and neomycin. The effect of antibiotics on intestinal microflora will be examined by quantitative analysis of aerobic and anaerobic bacteria and yeasts present in faecal pellets. No major side effects are expected from the administration of antibiotics in drinking water. However, animals will be closely monitored, and their body weight regularly recorded. *For flora reconstitution* - Prior to colonisation, faecal pellets used to reconstitute bacterial populations and the efficiency of bacteria inoculation will be confirmed by analysing the genomic DNA of total bacteria in faecal samples by qRT-PCR.

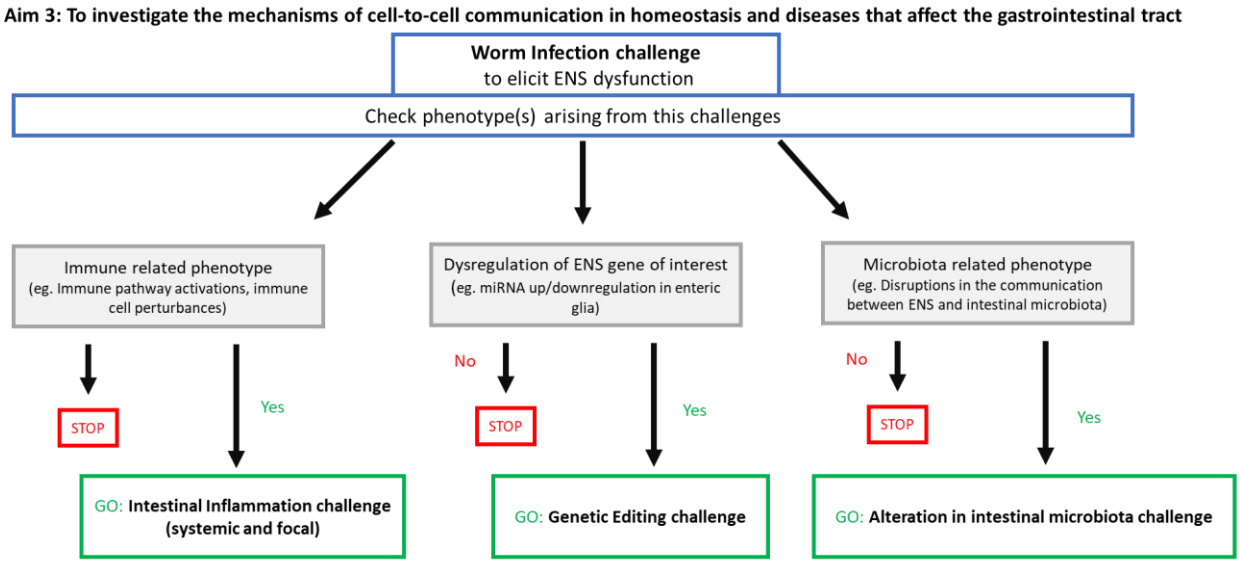

Figure 2. *Flowchart depicting crucial Go/No-go decision moments.*

NB – Only dysregulated systems will be studied further. So, if dysregulation in multiple systems (the immune system, and/or the microbiome and/or ENS target genes) arises from the worm infection challenge then animals can be subjected to more than one follow-up challenge (e.g. intestinal inflammation challenge and alteration in intestinal microbiota challenge).

OUTCOME MEASUREMENTS THAT WILL BE ANALYSED

As mentioned above, we will perform *in vivo* and/or *ex vivo* (post-mortem) analysis of the following outcome measures (the experimental methodologies that will be used are included as well). **Rationale: these outcome measures are included because they are ideally suited to reflect on the status and composition of the ENS, its modes of communication with, and effect on, surrounding tissues and cellular systems (e.g. microbiota), and its role in regulating gastrointestinal homeostasis (please also see background of Project Proposal).**

NB – Many of the outcome parameters can be analysed by using the same group of animals (e.g., ENS structure, intercellular communication, gut physiology microbiota analysis). Support on the group sizes and total number of animals can be found at statistical methods and section B. Animals – Number (where we refer to the following Word document: Total number of mice;

table – appendix 3). As explained before, the provided examples of mouse models in these tables are at present the preferable models, but we will substitute them if better models become available.

- ***ENS structure/ (epi) genetic signature & ENS function & ENS proliferation and survival***

Distinct imaging techniques will be used to study these parameters on ENS:

We will examine the influence of challenges mentioned above (e.g., worm infection, genetic editing of the ENS, intestinal inflammation, alteration in intestinal microbiota) on ENS composition, architecture, morphology and activity (**ex vivo)**.

a) ***Fate mapping experiments*** - we will use *Sox10.CreER^{T2}:R26td^{Tomato}* in challenge models described above. Tamoxifen (i.p.) will be injected at the time of initiation of the challenging protocol to label Sox10 expressing EGCs with tdTomato reporter. **– 12 animals per group.**

b) ***Calcium imaging of ENS activity*** - We will examine the ENS activity *ex vivo* in different ways: i) [Ca2+]i-Fluo-4 imaging: Live recording of ENS activity will be performed using Fluo-4 Ca2+ -imaging in preparations of whole-mount mouse gut after various stimulations (chemical, electrical). ii) For the other Ca2+ imaging experiments we will use Sox10-CreERT2::R26-GCaMP6f mice (EGCs, tamoxifen injection in adult animals) and Wnt1.Cre:: R26-GCaMP6f mice (all enteric neurons and glia). **– 12 animals per group**

c) ***For cell proliferation and survival:*** Analysis of ENS proliferation at different stages of the challenges detailed above. Animals will be given a thymidine analogue (e.g. BrdU, edU) to label cycling cells via i.p. injections and/or drinking water. In vivo/ex vivo labelling assays will be used to detect cell death, senescence, DNA damage. We will perform this experiment only if we observe any phenotype in organisation, composition and function of the ENS. – **12 animals per group**

We will investigate the molecular signature of ENS cells and/or extracellular components in gut homeostasis and under intestinal challenge. We will use FACS to isolate ENS cells for qRT-PCR, bulk or single-cell RNA Sequencing

d) ***Tissue isolation, digestion and FACS***: Intestinal preparations will be dissociated into single cell suspensions and subjected to FACS for isolation of ENS cells and/or other components and analysis by qRT-PCR, bulk and single cell RNA Sequencing. **- 9 animals per group**

- ***Intercellular communication – 6 and 9 animals per group***

We will investigate cell-to-cell communication between ENS cells and other intestinal and extra-intestinal systems by immunohistochemistry, qRT-PCR, western blotting, ELISA, etc. Mice that underwent intestinal challenge (specified above) will be euthanised and intestinal and extra intestinal tissues will be collected for analysis. **NB: The tissue collection will be performed separately only when it cannot be achieved together with other experiments, in order to minimise unnecessary culling and animal use.**

- ***Gut physiology (intestinal motility & gut permeability)***

To study intestinal motility ***in vivo*** we will implement different methods:

a) ***Total intestinal transit time*****:** The protocol for the total gastrointestinal transit time will be performed as previously described[5]. Mice will be individually placed into cages devoid of bedding and fasted for an hour. Next, a dye will be administrated by oral gavage, and the animals returned to their individual cages. The total intestinal transit time will be calculated by the time from gavage until the extrusion of the first coloured pellet. A maximum period of observation will be 5 hours in all experiments and mice that fail to expel the coloured pellet within this time will be quantified as ending point at 5 hours. ***NB: No culling is needed, and mice can be used in other read-out parameters. The dye is cleared from the gastrointestinal tract 2 days after administration. Faeces will be collected and analysed for bacterial composition and stool quality.**

b) ***Small intestinal transit:*** As described for the total transit time, mice will be given dye via oral gavage. Mice will be euthanised, followed by removal of the small intestine. The total length of the small intestine and the distance that the dye has travelled will be measured to determine the small intestinal transit length. ***Faeces will be collected**

and analysed for bacterial composition and stool quality. Tissues will be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain).

c) **Colonic propulsion**: Colonic propulsion will be evaluated by first lightly anesthetizing the animals with isoflurane. A small diameter glass bead will be inserted into the rectum, and the expulsion time of the glass bead will be recorded. The assay will be repeated twice every 100 min for a total of 3 bead insertions. ***NB: No culling is needed, and mice can be used in other read-outs.**

NB – experiments in a and c can be performed using the same group of animals – 12 animals per group.

In case of a phenotype in a, b and/or c - To study the intestinal contractility and motility ***ex vivo*** using live video recording and spatiotemporal analyses of ENS-dependent motility. Experiments can be performed using tissue derived from animals used in b.

d) Segments from distinct parts of the gut of young adult mice will be removed as previously described[6]. Segments will be carefully isolated, luminal contents emptied and placed loosely pinned onto an organ bath chamber continuously infused with Krebs solution and constantly supplied with 95% O2 and 5% CO2 at 37°C and the neurogenic intestinal motility recorded.

To study gut permeability ***in vivo***:

e) *Measuring levels of plasma FITC:* - Intestinal permeability will be determined by measuring levels of plasma FITC after administration via oral gavage of FITC-conjugated dextran in PBS. Blood will be obtained after administration, and the concentration of fluorescein will be determined by spectrophotofluorometry. Mice will be euthanized during this experiment. ***Faeces can be collected and analysed for bacterial composition and stool quality. Tissues can be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain)._– 12 animals per group**

- ***Vesicle trafficking analysis***

Distinct imaging techniques will be used to study ENS vesicle trafficking **– 12 animals per group**:

a) FM1-43 imaging ***(in vitro)***: We will investigate how synaptic signalling and synaptic vesicle turnover (FM1-43) differ in ENS cultures derived from the different mouse models by using FM1-43 imaging and after distinct stimuli (chemical, electrical).
b) NTA analysis ***(in vitro*** and ***ex vivo)*** - To analyse pattern parameters of EVs: intensity fluctuations, surface geometry and shape of the particles as well as particle concentration to distinguish sub-populations of vesicles.
c) Manipulation of EVs by viral vector targeting in the ENS ***(in vivo)*** - AAVx.XFP carrying genetic modifiers to silence (shmiRNA) or overexpress molecules of interest (miRNAs), will be injected (i.v.) in our animal models to evaluate the role of EVs in the maintenance, organisation and function of the ENS. No side effects are expected with AAV injections (mild discomfort). ***NB – Most of these experiments described above can be performed using the same group of animals as they are likely to cause transient and mild pain and discomfort.**

- ***Inflammatory processes and immunophenotyping***

Gut inflammation will also be closely monitored ***in vivo*** (faecal analysis) and scored ***ex vivo*** by using **immunohistochemical** staining for inflammatory markers and immune cells, **H&E** and **flow cytometry (see below).** For the immunophenotyping, intestinal, lymphoid tissues and blood preparations will be dissociated, and single cell suspensions counted and pre-incubated with antibodies to label myeloid and lymphoid cells. Stained samples will be processed using a flow cytometer, and the obtained raw data analysed. Different cell populations will be identified by the general gating strategy and the total number of cells estimated by the cell count multiplied by the percentage of live cells- ***12 animals per group/timepoint*** ***NB These experiments can be performed using the same group of animals.**

- ***Microbiota analysis***

Metagenomic DNA extraction from faecal, and tissue samples and molecular profiling/16S rRNA sequencing will be carried out to explore differences in the microbial community structure between experimental groups. We will examine the differences in microbial richness (e.g., Chao1) and diversity (e.g., Shannon). ***NB: The tissue collection will be achieved together with other experiments, in order to minimise unnecessary culling and animal use, so no extra animals will be used for this outcome measure.**

- ***Development/progression/resolution of infection***

For **experiments using the worm infection model,** we will closely track worm burden, numbers and eggs at different time points in vivo. **NB: No need for euthanasia, so these experiments will be performed together with the ex vivo analysis of tissue and faeces under "Inflammatory processes and immunophenotyping" to following the infection and inflammation.**

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

For the quantitative experiments, design has been based on ARRIVE guidelines and sample sizes were set using power analysis https://www.statstodo.com/SSizCorr_Pgm.php. to determine the number of mice needed for each experiment, generally considering a difference between groups of at least 20%, power of 80% and significance level of 5%.
For qualitative experiments, we will use the minimum number of mice to provide an accurate description based on previous publications and on our own experience.

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
1	mouse	In house breeding	(Young) adults	2385* (see below)	Male and female	Wildtype, Mice with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells, Transgenic mice with altered ENS, Specifically modulate ENS activity	

Provide justifications for these choices

Species	Mice are used due to their anatomical, physiological, and genetic similarity to humans as well as their small size, ease of maintenance, short life cycle, and abundant genetic resources.
Origin	Sox10.CreERT2:R26tdTomato from a licensed non-commercial breeder in UK (but currently in house), Sox10.CreERT2:R26-GCaMP6f and *Wnt1.Cre:R26-hM4Di-DREADD* from licensed non-commercial breeders within Europe,NDRG4$^{fl/fl}$;Wnt1.Cre, NSE-Noggn and Hand2$^{fl/+}$;Wnt1.Cre are all in house, CD63 floxed from a licensed non-commercial breeder in USA
Life stages	Young adults (1-10 months) is most apt for optimal results

Number	Based on the literature, on our own and other collaborators experiences, we estimate that we will need a maximum of 12 mice per group (high amount of altered tissue needed and/or more variability/spread expected because of the measurement method (for example, gut transit times vary a lot depending also on whether mice are very active or passive at the time of the experiment; inflammation development/progression is also very variable (unpublished data in collaboration with Francis Crick Institute))). For FACS experiments, we will need max 9 mice per group (less material needed, lower variability (unpublished data)) and 6 mice for RNA experiments (less material needed, low drop-out expected and also low spread expected; RNA can partly also be obtained together with other outcome parameters (unpublished data; 5.1 Ild2e, 5. et al 2017; 5.1 Ild2e, 5. et al 2021)). Total for all lines/groups and all outcome measures, this will be a maximum of 954 animals for the worm infection model alone. For a more detailed overview of how this number is built up, we refer to the following Word document: Total number of mice; table – appendix 3. If dysregulation in the immune system, and/or the microbiome and/or ENS target genes arise from the worm infection challenge then animals can be subjected one or more of the follow-up challenges (e.g. intestinal inflammation challenge and alteration in intestinal microbiota challenge), which requires a similar number of outcome measures to be used for these models (**go/no-go moment**). However, we will choose specific models to continue on the other challenges/models (based on the effects in the worm infection model), so we will use maximally 2 mouse lines instead of 4 for follow-up challenges, which means a maximum of 477 mice per challenge. If all challenges/models will be a go (=3 extra), this will maximally lead to 1431 (= 3 x 477) more animals. * A total of maximum 2385 (= 954 + 1431) mice will be used in this appendix including a maximum of all go/no-go animals. In case certain phenotypes are observed in experiments using these lines, other mouse strains can also be added to the same experimental set up to analyse more detailed pathways (lines to specifically study ENS activity, EVs, miRNAs - see list below). ***NB - All genotypes that are not going to be used for the procedures described above can be used in appendix one for culturing cells and tissues, to follow the principles of 3Rs.**
Gender	Both male and female mice will be used to minimise any gender-related variations in the experiment.
Genetic alterations	Mice with fluorescently labelled ENS (e.g. Sox10.CreERT2:R26tdTomato, Wnt1.Cre:R26tdTomato, Sox10.CreERT2:R26-GCaMP6f) will be used to investigate the ENS in depth. Specific ENS cells will be labelled and can thereby be easily followed in these models. Furthermore, activity and specific ENS cells can be studied. This will give us more insight into the function and activity of ENS cells and the interactions between these cell types. Mice with fluorescently labelled extracellular vesicles from ENS cells (e.g. $CD63^{X/X}$ crossed with ENS specific cre reporter lines e.g., $Sox10.CreER^{T2}$ or Wnt1.Cre) can be used to study the involvement of extracellular vesicles in the interaction between the ENS and it surrounding tissues and systems. Lines designed to specifically modulate ENS activity (e.g. Wnt1.Cre:R26-hM4Di-DREADD). **(**This line can (but not necessarily will) be used in case of appearance of specific phenotypes related to ENS activity); Mice with a knockdown/overexpression of miRNAs in ENS cells (e.g injected with +AAVx:ENS-specific cre) and/or knockout mice for specific miRNAs that are relevant for gastrointestinal function. Sox10.CreERT2:R26tdTomato will be injected with cell-specific cre dependent +AAVx vector (eg. miRNAs in enteric neurons) in case of a strong phenotype related to miRNA manipulation in *in vitro assays*. Alltogether, these models will help us unravel the interface of the adult ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal tissues (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (microbiota) in health and may lead to possible pathways and/or targets that could be used for treatment in the future.
Strain	(mixed) C57BL/6J (e.g. Sox10.CreERT2, R26-hM4Di-DREADD, R26-GCaMP6f), (mixed) 129S4SvJaeJ (e.g. Wnt1.Cre). The strain of the wildtype mice will be matched to the corresponding transgenic mise used in the procedure.

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

The H.polygyrus is a gastrointestinal worm that is only transmitted orally by ingesting infective larvae (Camberis, Le Gros and Urban, 2003). For this reason, infected mice are housed in cages separate from non-infected mice. Trigene is used to wipe down cages before putting them back in the IVC racks as extra precautions.

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.

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

For the inflammation, surgical models and worm infection, animals might experience pain. To minimise pain, suffering and distress, we will use appropriate analgesia (e.g. opiates (not in experiments of gut motility) or NSAIDs (not in experiments involving inflammation)) and anaesthesia (e.g. isoflurane) that do not affect the normal intestinal homeostasis. However, use of analgesia is not expected based on experience with our past experiments, but pain will always be scored and evaluated to ensure proper management of the animals (if we cannot apply analgesia and animals experience discomfort, HEPs will be applied when necessary). Animals will be constantly monitored as further described in their respective program of work for each experimental procedure to immediately detect any signs of discomfort. Any animals that undergo any experimental procedures and/or manipulation will be closely and often monitored. All other challenges are not predicted to cause the animals any great or long-lasting harm.

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

- *In vivo* experiments can cause distress or mild to moderate discomfort to the animals.
- All animals will be frequently monitored for any signs of pain and distress. Changes to normal and provoked behaviour, movement, physical signs such as posture, respiration, skin and coat changes, inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persists for longer than 24 hours. Any mice exhibiting any deviation from the normal health and behaviour will be further monitored and treated.
- Disease and antibiotic models used in this protocol can cause animals to show sudden body weight loss (of 15% of their body weight).
- Inflammation mouse models can develop side effects, such as pain, weight loss, diarrhoea/constipation and anal bleedings (no ulcers with perforation are expected).
- *H. poly* are mouse pathogens and while wildtype mice are able to clear the worm without exhibiting significant clinical problems, mice carrying mutations may be more susceptible to pathology. All efforts will be made to minimise these symptoms, but it will be necessary to keep animals long enough to achieve measurable responses. Mice challenged with *H. poly* will be monitored for the appearance of clinical signs related to gut inflammation, including diarrhoea, blood stools, rectal prolapse, abdominal discomfort, bloating and weight loss. Infected mice will be observed and weighed during the first week of infection and when new mouse strains are used. Some weight loss is expected and this might be aggravated in mice carrying specific genetic mutations.

Explain why these effects may emerge.

- Most of the side effects mentioned above are unlikely in our non-diseased mice models (unless provoked), but can happen due to inflammation and/or progression of disease, and alteration in intestinal microbiota.

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

Animals will be frequently monitored by experienced staff and researchers. Our mouse colonies will be housed and maintained according to the basic guidelines for animal welfare. Working protocols will be adequately adjusted to benefit not only the research but also animal welfare. Experimental procedures/routes of administration, techniques that cause the least pain, suffering, distress and the shortest lasting harm will be preferred. Experimental procedures will be performed by using aseptic/sterile techniques, and drugs such as analgesics, anaesthetics and antibiotics will be administered whenever necessary. Examples of measures that we will use to prevent or minimise harm are extra hydration in case of diarrhea and boosting gels in case of high weight loss. Measures will be taken accordingly in the case any unexpected adverse effects might appear and animals will be immediately euthanized and experiments stopped in the case of unexpected severe signs of pain and discomfort.

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.

- All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persists for longer than 24 hours.
- Any animal will be immediately euthanised if it exhibit signs of suffering that is not transient or treatable and compromises its normal behaviour.
- Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe disomfort and >20% for severe discomfort) that persist for 48 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision.
- Genetically altered mice used in this appendix are not expected to show any deleterious phenotype, but in case they do, they will be immediately killed.
- Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled.
- Inflammation models: Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 3), "Wasting Syndrome" (body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), chronic diarrhea or opportunist infections, the animals will be immediately euthanised.
- In the case severe discomfort occur in multiple mice during the experimental procedures described abobe, the experiments will be ended and the animals euthanised.
- *H. poly* - Some weight loss is expected but mice carrying other mutations may show more severe responses. Any mice showing weight loss of 15% of starting weight or diarrhoea, bloody stools, rectal prolapse, abdominal discomfort and bloating for more than 24h will be killed.

Indicate the likely incidence.

For the *H. Poly* and other inflammatory/perturbed models (±10-15% incidence). The animals will be well monitored by experienced people, so that we are able to rapidly detect unforeseen adverse effects in early stages, whereby we aim to avoid reaching the humane endpoints and unexpected drop out.

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).

Appendix 3	Non-diseased gut	H. Poly infection model	Inflammation/perturbed models
Discomfort model itself → Cumulative discomfort ↓	**no discomfort**	**mild discomfort**	**moderate discomfort**
ENS structure /function /proliferation - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Gut physiology - Administration of substances: **mild** - Transit/motility assays: **mild** - Killing: **mild**	Mild	Moderate	Moderate
Vesicle trafficking - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Microbiota analysis - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Intercellular communication - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Inflammatory processes and immunophenotyping - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Moderate
Development/progression of worm infection - Administration of substances: **mild** - In vivo tracking: **mild**	Mild	Mild	Moderate

Figure 3: Cumulative discomfort for disease models (columns) combined with outcome measurements (rows). The different mouse lines itself are not expected to have any harmful phenotype or display signs of discomfort. Inflammation/perturbed models cover the models that might be used after the Go/No-go moment, including DSS/BAC treatment, (epi)genetic editing and microbiota alterations, and are therefore scaled as moderate discomfort.

Approximate percentages expected cumulative discomfort based on animal numbers per outcome measure and disease model:
Mild: 65%
Moderate: 35%
Severe: 0%

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	To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models, for instance the ones described in this document (such as H. poly infection models). In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied *in vivo*. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established *in vitro* culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis *in vivo.* We are also taking advantage of recently established collaborations in the gastroenterology clinic (5.1 lid2h to obtain human intestinal specimens (IBDs and IBS) and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study disease development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Reduction	Before embarking on any procedures in animal research, we are collecting as many evidence as it is necessary to determine whether a candidate gene or molecule also provide insights on *in vivo* pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This is also can be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals. This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.
Refinement	Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no clear phenotype and results, experiment that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached. Discomfort level in inflammatory, *H. poly* models might be mild to moderate (DSS). In the case of severe discomfort, mice will be immediately sacrificed.

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.

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.

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.

NA

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.

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.

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

Ex vivo (post-mortem) and *in vitro* experiments will be performed in tissue and cells collected from our experimental groups to further analyse the biology of the nervous system.

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.

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

☒ No > Describe the method of killing.

Cervical dislocation

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

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

Appendix 3	Before Go/No-go moment					Maximum numbers after Go/No-go	
Non-diseased gut	***Sox10CreERT2/wnt1Cre-TdTomato (ctrl worm infection)***	***Sox10CreERT2-R26-GCaMP6f (ctrl worm infection)***	***CD63-Sox10CreERT2/wnt1Cre (ctrl worm infection)***	***Sox10CreERT2/wnt1Cre-R26-hM4Di-DREADD (ctrl worm infection)***	***Total***	***Max animals after Go/No-go (ctrl follow-up challenges) Further explained in appendix 3****	***Total***
ENS structure /function /proliferation	#/measure	#/measure	#/measure	#/measure		#/measure	
Number/morphology (a)	12	12	12	12		72	
Activity (b)	12	12	12	12		72	
Proliferation/survival (c)	12	12	12	12		72	
FACS (d)	9	9	9	9	180	54	270
Intercellular communication	#/measure	#/measure	#/measure	#/measure		#/measure	
RNA/ protein	6		6	6		27	
FACS	9		9	9	45	40	67
Gut physiology	#/measure	#/measure	#/measure	#/measure		#/measure	
Whole-gut transit + colonic (a,c)	12		12	12		54	
Small intestinal transit + ex vivo motiliy (b,d)	12		12	12		54	
Permeability (e)	12		12	12	108	54	162
Vesicle trafficking	#/measure	#/measure	#/measure	#/measure		#/measure	
Imaging (a,b,c,d)	12		12	12	36	54	54
Inflammatory processes and immunophenotyping	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Faecal + tissue analysis	36		36	36	108	162	162
Microbiota analysis	#/measure	#/measure	#/measure	#/measure		#/measure	
Faecal + tissue analysis	With other		With other	With other	0	With other	0
Development/progression of worm infection	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Worm burden/number/eggs	With other		With other	With other		With other	
					0		0
*** See figure 2 for Go/No-go decisions. Animals will be subjected to 1 or more challenges following worm infection. Animal models: to be determined.**					477		715

Appendix 3	Before Go/No-go moment					Maximum numbers after Go/No-go	
Diseased gut	***Sox10CreERT2/wnt1Cre-TdTomato (worm infection)***	***Sox10CreERT2-R26-GCaMP6f (worm infection)***	***CD63-Sox10CreERT2/wnt1Cre (worm infection)***	***Sox10CreERT2/wnt1Cre-R26-hM4Di-DREADD (worm infection)***	***Total***	***Max animals after Go/No-go (follow-up challenges) Further explained in appendix 3****	***Total***
ENS structure /function /proliferation	#/measure	#/measure	#/measure	#/measure		#/measure	
Number/morphology (a)	12	12	12	12		72	
Activity (b)	12	12	12	12		72	
Proliferation/survival (c)	12	12	12	12		72	
FACS (e)	9	9	9	9	180	54	270
Intercellular communication	#/measure	#/measure	#/measure	#/measure		#/measure	
Histology/ RNA/ protein	6		6	6		27	
FACS	9		9	9	45	41	68
Gut physiology	#/measure	#/measure	#/measure	#/measure		#/measure	
Whole-gut transit + colonic (a,c)	12		12	12		54	
Small intestinal transit + ex vivo motility (b,d)	12		12	12		54	
Permeability (e)	12		12	12	108	54	162
Vesicle trafficking	#/measure	#/measure	#/measure	#/measure		#/measure	
Imaging (a,b,c,d)	12		12	12	36	54	54
Inflammatory processes and immunophenotyping	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Faecal + tissue analysis	36		36	36	108	162	162
Microbiota analysis	#/measure	#/measure	#/measure	#/measure		#/measure	
Faecal + tissue analysis	With other		With other	With other	0	With other	0
Development/progression of worm infection	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)	12/measure/timepoint (3 timepoints)		12/measure/timepoint (3 timepoints)	
Worm burden/number/eggs	With other		With other	With other		With other	
					0		0
*** See figure 2 for Go/No-go decisions. Animals will be subjected to 1 or more challenges following worm infection. Animal models: to be determined.**					477		716
Total animal number					2385		

NIET-TECHNISCHE PROJECTSAMENVATTING

Naam van het project	Communicatie tussen het darmzenuwstelsel (i.e. enterisch zenuwstelsel), darmcellen en andere weefselomgevingen: van darmevenwicht tot de ontwikkeling en uitgroei van verscheidene ziektes.
NTS-identificatiecode	NTS-NL-830095 v.1
Nationale identificatiecode van de NTS *Veld wordt niet gepubliceerd.*	
Land	Nederland
Taal	nl
Indiening bij EU *Veld wordt niet gepubliceerd.*	ja
Duur van het project, uitgedrukt in maanden.	60
Trefwoorden	enteric nervous system gastrointestinal tract crosstalk intestinal cancer cells epigenetics
Doel(en) van het project	Fundamenteel onderzoek: Oncologie Fundamenteel onderzoek: Zenuwstelsel Fundamenteel onderzoek: Gastro-intestinaal stelsel met inbegrip van de lever

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).	Het algemene doel van dit project is de organisatie en functie van het enterisch zenuwstelsel (EZS) tijdens ziekte en gezondheid te doorgronden en de interacties van dit buikbrein met andere organen en cellulaire systemen beter te begrijpen. Meer specifiek beogen we om met behulp van nieuwe modellen en reeds bestaande methodiek de communicatie tussen het EZS en andere celtypes te bestuderen, de cellulaire en moleculaire interacties in het EZS en tussen het EZS en andere cellulaire systemen te onderzoeken, en de neurale mechanismen die de normale en verstoorde (vb. darmontsteking, darmkanker) werking van het spijsverteringsstelsel reguleren bloot te leggen.
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 het project) en voordelen op lange termijn (die mogelijk pas worden bereikt nadat het project is afgerond).	Het is een hele uitdaging om de organisatie en werking van het buikbrein te doorgronden als gevolg van zijn ingewikkelde samenstelling en moeilijk bereikbare locatie in de darmwand. Echter, dankzij samenwerkingen met andere laboratoria heeft onze onderzoeksgroep de nodige kennis en methodologie om de cellulaire en moleculaire interacties in het EZS en tussen het EZS en andere cellulaire systemen in detail te onderzoeken. Door deze complexiteit en het groot aantal cellulaire systemen waarmee het EZS zijn activiteit integreert zullen de bevindingen van onze studies ook als basis kunnen dienen voor onderzoek naar andere orgaansystemen en het centraal zenuwstelsel. Huidig onderzoek toont ook aan dat de darm, en meer bepaald het EZS als een toegangsdeur kan optreden in het ontstaan van verschillende ziektes die niet erkend staan als aandoeningen van het gastro-intestinaal stelsel. Hiertoe behoren onder andere neurodegeneratieve en mentale aandoeningen van het centraal zenuwstelsel. Een beter begrip van de organisatie en functie van het EZS is uiteraard ook essentieel om de ontstaansmechanismen van een hele lijst van gastro-intestinale aandoeningen (enterische neuropathieën, functionele gastro-intestinale aandoeningen (vb. prikkelbaar darmsyndroom) en inflammatoire darmziektes (vb. de ziekte van Crohn) in detail te ontrafelen. Bovendien zijn er steeds meer aanwijzingen, inclusief deze vanuit ons eigen laboratorium, dat aantoont dat het EZS een rol speelt bij dikke darmkanker. Met onze onderzoeksstrategie, gecombineerd met ervaringen vanuit de kliniek van gastro-enterologen, trachten we cellulaire en moleculaire mechanismen te identificeren, en nieuwe detectie markers (i.e. biomarkers te vinden, waarmee het ontstaan en de progressie van ziekte kan worden verklaard. Verder zal ons onderzoek

	duidelijk maken in hoeverre en op welke wijze het EZS als therapeutisch doelwit kan worden gebruikt voor de hierboven aangehaalde ziektes.

VOORSPELDE SCHADE

In welke procedures worden de dieren gewoonlijk gebruikt (bijvoorbeeld injecties, chirurgische procedures)? Vermeld het aantal en de duur van deze procedures.	De muizen zullen enkele specifieke procedures ondergaan. Voor het eerste deel van ons onderzoek, het zogenaamde celkweek (in vitro) deel, zullen de dieren geen specifieke procedures ondergaan vooraleer we de darm isoleren om onze celkweek systemen op te zetten. Verder zullen we bepaalde ziektebeelden opwekken in de muizen. Dit zullen we op verschillende manieren aanpakken. We focussen daarbij op het nabootsen van dikke darmkanker en ontstekings-gerelateerde darmkanker. Deze ziektes kunnen we imiteren door bijv. een fout in het genetisch materiaal van de muizen te introduceren of door de muizen te injecteren met een kankerverwekkende stof, al dan niet in combinatie met een ontsteking verwekkende substantie. Indien hieruit blijkt dat de muizen succesvol tumoren ontwikkelen, zullen we pas over kunnen gaan tot het toepassen en bestuderen van de efficiëntie van behandelingen, zoals chemo- of radiotherapie. Daarnaast zullen we ons focussen op een darminfectie en afhankelijk van deze resultaten eventueel andere ontstekings-gerelateerde ziektebeelden nabootsen. De modellen zullen vervolgens onderworpen worden aan enkele in vivo analyses. Dit zijn analyses die we uitvoeren in de levende dieren, zoals vb. het bestuderen van darmfunctie/darmbeweging, maar voornamelijk aan ex vivo analyses (zogenaamde analyses met levend weefsels buiten het lichaam om vb. de structuur van het EZS, de ontwikkeling en progressie van kanker/ontsteking te bestuderen). Belangrijk te vermelden is dat we telkens de procedure uitvoeren die een zo minimaal en kort mogelijk effect heeft op pijn/ongemak van de dieren.
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?	We verwachten dat het grootste deel van de muizen geen tot mild ongerief ondervinden. Als gevolg van hun genetische afwijkingen in het enterisch zenuwstelsel, kunnen de muizen mogelijk last ondervinden van een veranderde darmbeweging. Wanneer de dieren onderworpen worden aan chirurgische procedures, procedures om stoffen te injecteren en/of de verscheidene ziektebeelden na te bootsen, zullen zij een mild tot matig ongerief kunnen ondervinden. Dit voornamelijk als gevolg van een verstoorde darmbeweging, ontstekingen, bloedverlies en gewichtsverlies. Tot slot kan er ook ernstig ongerief optreden op het ogenblik dat de muizen met een veranderd EZS onderworpen worden aan bepaalde ziektemodellen en behandeld worden met radio/chemotherapie, alsook wanneer ze gebruikt worden om de ontwikkeling en groei van tumoren live te volgen d.m.v. CT-scans.
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)	5825	0	3942	1343	540

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*

Geef de redenen voor het geplande lot van de dieren na de procedure.	Voor het beoordelen van verschillende darmfunctionaliteiten (bv. darmbeweging/ darmdoorlaatbaarheid) worden dezelfde dieren op verschillende tijdstippen onderworpen aan verschillende experimenten. Ook wanneer de tumor burden bepaald wordt, zullen dezelfde dieren op verschillende tijdstippen CT-scans ondergaan. Wanneer de dieren alle experimentele procedures volledig doorlopen hebben, zullen ze gedood worden en zal het materiaal van de overleden dieren gebruikt worden voor verder onderzoek.

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.	Aangezien we in de muismodellen de volledige fysiologie van het lichaam in acht kunnen nemen, zullen deze toelaten om de overkoepelende genetische en fysiologische mechanismen onderliggend aan de verschillende darmziektes te ontrafelen. Echter, om deze mechanismen volledig tot in detail uit te diepen zullen we gebruiken moeten maken van verschillende celkweekmodellen. Dit laat ons toe om op grotere en preciezere schaal de details te onderzoeken. Ons lab heeft ondertussen veel ervaring met het opzetten van deze celkweekmodellen. Dit laat toe om hierin kandidaatgenen/pathways te bestuderen, vooraleer we dit verder onderzoeken a.d.h.v. muizen. Daarnaast hebben we recentelijk ook nieuwe samenwerkingen in het 5.1 lid2h opgezet (bijvoorbeeld met de gastroenterology afdeling), waardoor we ook a.d.h.v. humaan materiaal reeds de betrokkenheid van het ENS in deze verschillende ziektebeelden kunnen onderzoeken. Echter kunnen we niet volledig afstappen van diergebruik, aangezien deze systemen de volledige complexiteit van het lichamelijk functioneren omvatten. Echter zullen we de meest optimale modellen gebruiken.
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 we beginnen aan onze dierexperimenten, zullen we informatie verzamelen a.d.h.v. celkweek, (publieke) data en in silico analyses, zodat we gerichtere experimenten kunnen opzetten. We zullen ook de meest optimale modellen gebruiken, waardoor we de hoeveelheid muizen kunnen verminderen. Indien de muizen niet de juiste waarneembare in of uitwendige kenmerken (zoals vb. tumorvorming) vertonen of de eerste experimenten niet gehoopte resultaten opleveren, zullen we niet verder gaan met deze modellen. Omdat we over verschillende onderzoeksprojecten gelijkaardige modellen en procedures gebruiken, kunnen we data interpoleren over de verschillende projecten, waardoor we ook minder dieren nodig hebben. We zullen proberen om onze fok zo efficient mogelijk op te zetten en er zo voor te zorgen dat we overbodige kweken en een onnodig overschot van nakomelingen vermijden. Indien dit niet mogelijk is, zullen we onze fokstrategie zo zorgvuldig mogelijk opzetten zodat we heterozygote/homozygote en wildtype nestgenoten (met zelfde leeftijd) kunnen gebruiken voor de experimenten. Indien er onvoldoende informatie beschikbaar is, zullen we 'pilot-experimenten' opzetten, met een beperkt aantal dieren, zodat onze experimentele opzet geoptimaliseerd is.
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 nieuwe verfijningstechnieken in gebruik te nemen.	De gekozen modellen en procedures zijn essentieel om nieuwe inzichten in humane pathologieen die het EZS aantasten. Door modellen te gebruiken waarbij veranderingen in het EZS op een tijdelijke en/of heel specifieke manier geïntroduceerd worden, zullen de bijwerkingen beperkt blijven. Alle protocollen, modellen en behandelingen die onder deze licentie worden gebruikt, zijn standaard en werden eerder en uitgebreid uitgevoerd door leden van ons laboratorium, medewerkers en/of goed gedocumenteerd in relevante literatuur. Pilotstudies zullen vooraf worden uitgevoerd om te bepalen wat de minimale dosis en blootstellingstijd is die een effectieve werking vertoont met minimale neveneffecten. Ook zullen we ervoor zorgen dat de dieren zo weinig mogelijk bijwerkingen/ stress ondervinden van de procedures. Een van de mogelijkheden hiertoe is de toediening van verdoving (keuze verdovingsmiddel zonder effect op het functioneren van de darm).
Licht de keuze van de soorten en de bijbehorende levensstadia toe	In dit onderzoeksproject is gekozen voor muizen, niet alleen omwille van hun gebruiksgemak (e.g. klein, groepshuisvesting, grote beschikbaarheid aan modellen), maar met name vanwege hun enorme anatomische, fysiologische, en genetische gelijkenis met mensen. Bovendien zijn er veelvuldig toegepaste en valide onderzoeksprotocollen voor het werken met cellen en weefsels van deze dieren en voor het werken met levende dieren. Er zal voornamelijk met volwassen dieren gewerkt worden, vanwege de beschikbaarheid van valide protocollen voor deze leeftijdscategorie

	voor onze onderzoeksdoelen, en vanwege de betere vertaalbaarheid naar de menselijke situatie. Driedimensionale celkweek modellen van het enterisch zenuwstelsel zullen verkregen worden uit muizenembryo's of pasgeborenen, zoals veelvuldig toegepast is in eerdere studies. De driedimensionale celkweek modellen kunnen langdurig gebruikt worden, waardoor er maar een beperkt aantal embryo's of pasgeborenen nodig zal zijn.

VOOR EEN BEOORDELING ACHTERAF GESELECTEERD PROJECT

Project geselecteerd voor BA?	nee
Termijn voor BA	
Reden voor de beoordeling achteraf	
Bevat ernstige procedures	
Maakt gebruik van niet-menselijke primaten	
Andere reden	
Toelichting van de andere reden voor de beoordeling achteraf	

AANVULLENDE VELDEN

Nationaal veld 1 *Veld wordt niet gepubliceerd.*	
Nationaal veld 2 *Veld wordt niet gepubliceerd.*	
Nationaal veld 3 *Veld wordt niet gepubliceerd.*	
Nationaal veld 4 *Veld wordt niet gepubliceerd.*	
Nationaal veld 5 *Veld wordt niet gepubliceerd.*	
Startdatum project *Veld wordt niet gepubliceerd.*	
Einddatum project *Veld wordt niet gepubliceerd.*	
Goedkeuringsdatum project *Veld wordt niet gepubliceerd.*	
ICD-code 1 *Veld wordt niet gepubliceerd.*	
ICD-code 2 *Veld wordt niet gepubliceerd.*	
ICD-code 3 *Veld wordt niet gepubliceerd.*	
Link naar de eerdere versie van de NTS buiten het EC-systeem	

Centrale Commissie Dierproeven

16

Advies aan CCD

Datum 31 mei 2022
Betreft Advies Secretariaat over Aanvraag projectvergunning Dierproeven AVD202215867

Instelling:	5.1 lid2h
Onderzoeker:	5.1 lid2e
Project:	The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease
Aanvraagnummer:	AVD202215867
Betreft:	Nieuwe aanvraag
Categorieën:	Fundamenteel onderzoek

1 Inzicht in aanvraag en de eventuele knelpunten en risico's

Proces	Er zijn geen vragen gesteld aan de DEC. Aan de aanvrager is gevraagd over de NTS: Uw aanvraag draait om het enterisch zenuwstelsel. Kunt u in de NTS helder uitleggen wat dit is? Daarnaast bevat de NTS veel lastige termen die niet begrijpelijk zullen zijn voor een leek. Kunt u de NTS beter navolgbaar maken? U benoemt bij vervanging het 5.1 lid2h De NTS dient echter anoniem te zijn. Kunt u het 5.1 lid2h uit de NTS halen? Over bijlage 2 van de dierproeven: Wanneer in bijlage 2 van de dierproeven uit de pilot waarin de dosis chemo-en radiotherapie wordt onderzocht blijkt dat ernstig ongerief wordt voorkomen dan zal dit een 'go' moment zijn. Hierdoor zullen de dieren in het aangevraagde onderzoek geen ernstig ongerief ondervinden. Kunt u bij bijlage 2 van de dierproeven bij F de classificatie van het cumulatieve ongerief in procenten aanpassen?

Overzicht van opmerkingen bij 20. AdviesNotaCCD_1_5.1 lid2e_Met opmerkingen.pdf

Pagina: 1

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 16:43:00

Als je toch vragen gaat stellen over de NTS zou ik ook vragen om "mild ongerief" te vervangen door "licht ongerief".

Nummer: 2 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 17:04:28

Vind je de diersoort goed onderbouwd?

Nummer: 3 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 17:06:26

voor de netheid kunnen ze in bijlage 2 onder C ook vermelden hoe lang de dieren gevast worden, dit lees ik hier nu niet

Naam proef	Diersoort	Stam	Aantal dieren	Herkomst
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres				
	Muizen (Mus musculus)		1.322	Dieren die voor onderzoek gefokt zijn
3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer				
	Muizen (Mus musculus)		2.118	Dieren die voor onderzoek gefokt zijn
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases				
	Muizen (Mus musculus)		2.385	Dieren die voor onderzoek gefokt zijn

Huisvesting en verzorging anders dan Bijlage III Richtlijn

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

Citaat: If mice have to undergo CT imaging, they need to be food deprived before performing the CT scan, so that no fecal pellets are present in the colon during imaging. Food will be given immediately when animals have undergone their CT scan.

Gebruik van mannelijke en vrouwelijke dieren

3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

Locatie uitvoering experimenten	- Alle proeven vinden plaats in een instelling van een vergunninghouder. - Er zijn geen problemen bekend met de vergunninghouder.

2 DEC advies

DEC-advies	Citaat vraag DEC aan de aanvrager (vraag 38): Waarom worden dieren aangevraagd voor experimenten die ook omschreven zijn binnen vorig PV, welke nog loopt? Worden de experimenten beschreven in vorig PV niet meer uitgevoerd, hoewel er nog een jaar looptijd is?

Pagina: 2

Nummer: 1 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 17:19:06

bij 3.4.3.3 hebben ze ook iets ingevuld maar dit wijkt volgens mij niet af van de richtlijn dus ik zou het inderdaad negeren

Citaat antwoord aanvrager (vraag 38): We hebben hier enkel dezelfde diermodellen aangevraagd als in ons vorige PV als er nieuwe outcome parameters zijn toegevoegd die niet in onze huidige PV staan of we hebben voor dezelfde uitcome parameters nieuwe diermodellen aangevraagd. Een uitzondering hierop zijn de dieren voor therapie. Deze experimenten zijn niet meer haalbaar in de lopende PV en zijn dus verplaatst naar deze aanvraag.

Citaat vraag DEC aan de aanvrager (vraag 47): 10 CTs leidt tot een cumulatieve bestralingsdosis (whole body) van minimaal 3 Gy. Welk effect heeft dit op de uitleesparameters van de desbetreffende experimenten? En op het ongerief? Wat is de stralingsgevoeligheid van de muizenstammen die u wilt gebruiken?

Citaat antwoord aanvrager (vraag 47): Ongerief zal zoals beschreven, moderate-to-severe zijn. De pilootexperimenten die onder de huidige PV beschreven staan, zullen echter gebruikt worden om het effect van de therapie te bestuderen en het ongerief zoveel mogelijk te beperken. Om de stralingsgevoeligheid, de dosis en frequentie te bepalen zullen we samenwerken met andere onderzoekers die vergelijkbare experimenten eerder hebben uitgevoerd.

Citaat vraag DEC aan de aanvrager (vraag 53): U geeft aan dat bestraling en behandeling met chemotherapie van de muizen tot "moderate (to severe)' ongerief kan leiden. Verwacht u hier maximaal ernstig of maximaal matig ongerief?

Citaat antwoord aanvrager (vraag 53): We hebben hier op dit moment nog maximaal ernstig ongerief beschreven. We gaan echter met de pilootstudies onder onze huidige PV deze procedures optimaliseren en daarmee verwachten we, maar zijn we niet geheel zeker dat, het ongerief terug te kunnen brengen naar matig ongerief.

Citaat vraag DEC aan de aanvrager (vraag 4, ronde 2): De 5.1 lid2h kan geen ethische inschatting maken over het therapie gedeelte (ongerief, humane eindpunten) aangezien wordt aangegeven dat dit afhankelijk is van de uitkomst van pilootstudies die nog dienen te gebeuren op het huidig lopende PV (en daarom nu als maximaal ernstig wordt ingeschat). Echter op vraag 38 geeft u als antwoord dat alle therapie studies van huidig lopend PV niet meer zullen uitgevoerd worden en daarom zijn meegenomen in dit PV. Dit lijkt dus een belangrijke tegenstrijdigheid te zijn. Bovendien is het feit dat dieren ernstig ongerief kunnen ondervinden een belangrijk ethisch dilemma in deze aanvraag. Daardoor kan de

5.1 lid2h over dit gedeelte nu geen beslissing nemen. We stellen daarom voor om OF deze pilootstudies mee te nemen in huidige aanvraag met een duidelijke go/no-go beslissing naar de eigenlijke therapie studies (inclusief zo veel mogelijke beperking van het ongerief) OF de therapie-studies uit de aanvraag te halen.

Citaat antwoord aanvrager (vraag 4, ronde 2): Zoals correct opgemerkt zullen enkel de pilootstudies, maar niet de officiële therapiestudies uitgevoerd worden op het lopende PV. Deze huidige PV loopt namelijk nog 1,5 jaar, waarin wij verwachten deze pilootstudies uit te voeren. Daarom zijn deze pilootstudies dus niet meer opgenomen in deze nieuwe PV aanvraag. De therapiestudies zelf krijgen we niet meer uitgevoerd in de komende 1,5 jaar, waardoor deze wel opgenomen zijn in deze nieuwe PV. Om ervoor te zorgen dat er een goede ethische overweging gemaakt kan worden hebben we nu een duidelijker go/no-go moment toegevoegd (appendix 2, p.5). De pilootstudies uit de lopende PV zullen als go/no-go moment gelden voor de therapiestudies in deze PV. In het geval het niet mogelijk blijkt (op basis van de pilootstudies) om de therapiestudies met matig ongerief uit te voeren, zullen deze experimenten vervallen (max 432 dieren). Het ongerief zal hierdoor zoveel mogelijk beperkt worden en wordt geschat op moderate.

Citaat C10: Een deel van de dieren zal maximaal 16 uur zonder voedsel gezet worden, zodat er geen voedsel in de darmen zit bij het uitvoeren van de CT-scans. De 5.1 lid2h is er verder van verzekerd dat voldaan wordt aan huisvesting en verzorging volgens de richtlijn op basis van de daartoe strekkende verklaring (in duplo) van zowel de vertegenwoordiger van de vergunninghouder, als de aanvrager onder respectievelijk punt 6 der ondertekening van de aanvraag en punt F in de bijlagen.

Citaat C11: De 5.1 lid2h acht het ongerief grotendeels realistisch ingeschat. In het voorstel wordt aangegeven dat in totaal 68% van de dieren licht, 23% matig en 9% ernstig ongerief zal kunnen ervaren. De 5.1 lid2h vindt dat ernstig ongerief binnen deze aanvraag niet gerechtvaardigd is. Uit de antwoorden van de onderzoekers (vraag 4, tweede ronde) is namelijk gebleken dat er op een lopende vergunning pilotstudies uitgevoerd zullen worden waarbij de optimale omstandigheden voor de experimenten met potentieel ernstig ongerief bepaald worden. Wanneer de uitkomst van de pilotstudies is dat er ernstig ongerief zal optreden, is dit aangemerkt als een 'no-go' voor de betreffende vervolg-experimenten binnen deze aanvraag.

Ethische afweging van de DEC:
Citaat:

	1. Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan? 2. -Waarden die voor de proefdieren in het geding zijn: substantieel nadeel -Waarden die voor onderzoekers bevorderd worden: reëel voordeel. -Waarden die voor de medische wetenschap bevorderd worden: reëel voordeel. De 5.1 lid2h is van mening dat de belangen van onderzoekers/wetenschappelijke instituten, binnen het project 'The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease' zwaarder wegen dan de belangen/waarden van de proefdieren/dierbeschermingsorganisaties, mits ernstig ongerief wordt voorkomen. Voor de betrokken proefdieren leiden de beschreven proeven tot de dood na, in het voorstel aangegeven licht ongerief voor 68% van de dieren, matig ongerief voor 23% van de dieren en ernstig ongerief voor 9% van de dieren. De 5.1 lid2h is echter van mening dat ernstig ongerief voorkomen dient te worden, omdat er een go-no go is ingebouwd door de onderzoekers tussen deze aanvraag en een reeds lopende vergunning. Wanneer uit de pilotstudies onder de lopende vergunning blijkt dat ernstig ongerief niet vermeden kan worden, zullen de betreffende experimenten in de huidige aanvraag geen doorgang vinden (zie antwoord op vraag 4 uit de tweede ronde vragen). De dieren worden door de experimenten in hun welzijn geschaad. De integriteit van de dieren zal worden aangetast door de experimentele handelingen (injecties (o.a. voor het induceren van genetische veranderingen en het toedienen van chemische middelen), inductie van darmkanker (via injecties, of via colonoscopie), CT-scans (met bijbehorende voedseldeprivatie), bestraling, chemotherapie, verschillende manieren voor het induceren van darminflammatie (worminfectie, dextraan natriumsulfaat (DSS)), laparotomie, het veranderen van de darmflora (antibioticumkuur gevolgd door fecaal transplantaat, vasten) en het leven met de gevolgen daarvan gedurende de proeven en de opoffering aan het eind daarvan. Indien de doelstellingen bereikt worden, zal dit project echter leiden tot meer inzicht in de communicatie tussen het enterische zenuwstelsel en andere celtypen in homeostase of onder pathologische omstandigheden

zoals inflammatie, kanker of veranderingen in de microbiota. Daardoor zullen aandoeningen aan de darm mogelijk beter begrepen worden en worden deuren geopend naar nieuwe interventies.

Het is aannemelijk dat de doelstelling behaald zal worden. De onderzoekers zullen zoveel mogelijk trachten het lijden van de dieren te beperken.

3. De 5.1 lid2h beantwoordt de centrale morele vraag 'Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan?' bevestigend.

De 5.1 lid2h onderschrijft de integriteit en intrinsieke waarde van het dier en heeft oog voor het te ondergane ongerief van de proefdieren. Naar haar mening weegt het reële belang van dit project, en meer specifiek de belangen van de onderzoekers en het betreffende onderzoeksgebied zwaarder dan de voorgestelde schending van integriteit, het te berokkenen ongerief en opoffering, mits ernstig ongerief wordt vermeden.

De 5.1 lid2h is van mening dat de voorgestelde experimentele opzet en de uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en dat de gekozen strategie en de voorgestelde experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het programma. De onderzoekers beschikken over de benodigde kennis en technische expertise, zoals duidelijk uit hun voorstel blijkt. Er is geen sprake van duplicatie.

In de gekozen strategie wordt op bevredigende wijze tegemoetgekomen aan de vereisten van vervanging, vermindering en verfijning. De 5.1 lid2h is ervan overtuigd dat de aanvrager voldoende maatregelen treft om zowel het ongerief van de dieren als het aantal benodigde dieren tot een minimum te beperken. Er zijn voldoende go/no-go momenten voorzien om onnodige dierproeven te vermijden. De 5.1 lid2h s ervan overtuigd dat er geen alternatieven zijn, waardoor deze dierproef met minder ongerief of met minder, dan wel zonder levende dieren zou kunnen worden uitgevoerd. Wel is zij van mening dat ernstig ongerief vermeden kan en moet worden.

Op grond van deze overwegingen beschouwt de 5.1 lid2h de voorgestelde dierproeven in het projectvoorstel "The crosstalk between the enteric

	nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" als ethisch gerechtvaardigd, mits ernstig ongerief wordt vermeden. Derhalve voorziet de 5.1 lid2h het projectvoorstel "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" van een positief advies onder voorwaarde dat ernstig ongerief voor de dieren vermeden zal worden. De DEC heeft extern advies ingewonnen bij - de aanvrager is om aanvullingen gevraagd De DEC heeft een groot aantal vragen gesteld, opgenomen in 2 bijlagen van het DEC advies. Het DEC advies is Verlenen onder voorwaarden De onderzoekers geven aan dat uitkomsten van pilotstudies op een lopende vergunning zorgen voor een go-no go voor het deel van de experimenten die hier worden aangevraagd. Daarbij is een no-go van toepassing wanneer uit de pilot-experimenten blijkt dat ernstig ongerief niet vermeden kan worden. Het uitgebrachte advies is niet gebaseerd op consensus. Citaat: Het uitgebrachte 5.1 lid2h advies is tot stand gekomen op basis van een meerderheidsstandpunt. Een DEC-lid nam een minderheidsstandpunt in. De overwegingen hiervoor waren: (1) het grote aantal muizen staat nauwelijks in verhouding tot de praktische baten van het onderzoek; aangezien het fundamenteel onderzoek betreft is het onzeker of hier nieuwe therapieën uit voort gaan komen; (2) het gebruik van genetisch gemodificeerde muizen. De volgende dilemma's zijn gesignaleerd door de DEC: Citaat: Het uitgebrachte 5.1 lid2h advies is tot stand gekomen op basis van een meerderheidsstandpunt. Een DEC-lid nam een minderheidsstandpunt in. De overwegingen hiervoor waren: (1) het grote aantal muizen staat nauwelijks in verhouding tot de praktische baten van het onderzoek; aangezien het fundamenteel onderzoek betreft is het onzeker of hier nieuwe therapieën uit voort gaan komen; (2) het gebruik van genetisch gemodificeerde muizen.

3 Kwaliteit DEC advies

Kwaliteit DEC-advies	
Het DEC advies is helder en navolgbaar. Bij de beantwoording van de beoordelingsvragen verstrekt u een heldere onderbouwing. De CCD had bij vraag C10 graag de mening van de DEC gezien. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. Het valt op dat u zeer veel vragen hebt gesteld. Hoewel dit de kwaliteit van de aanvraag verbetert, wekt het ook de suggestie van meeschrijven, wat onwenselijk is. U heeft in uw advies een voorwaarde voorgesteld. Het is de CCD duidelijk waarom u de voorgestelde voorwaarde wilt stellen. De CCD stelt het op prijs dat goed navolgbaar in uw advies is opgenomen dat het advies is gebaseerd op een meerderheidsstandpunt, en waarop het minderheidsstandpunt op gebaseerd is. Ook stelt de CCD het op prijs dat bij E3 goed navolgbaar is weergegeven waar discussie over is geweest tijdens de bespreking van deze aanvraag.	

4 Inhoudelijke beoordeling

Doelstelling Doelstelling	Citaat: The ultimate goal of this project is to get a better understanding of the molecular pathways that orchestrate the organisation and function of the ENS, and its integration with other tissues (e.g., brain) and systems (e.g., immune, vascular and epithelial systems) during physiological challenges (e.g. microbiota) and pathological challenges (e.g. gastrointestinal diseases like cancer, worm infection). This will bring us closer to understand how the ENS participates in the onset and progression of diseases that affect the gastrointestinal tract, and therefore will eventually contribute to the development of new targets and therapies to tackle gastrointestinal disorders. For this purpose, we aim to develop a holistic and advanced approach to study the influence of the intestinal environment on ENS transcriptome, morphology and physiology, both in health and disease, for which we will use well-established murine models and procedures.
Wetenschappelijk en maatschappelijk belang	Citaat: Relevance for research: Research interest focusing on the gut has increased in recent years because of the clinical and biological relevance of this organ in several diseases. More specifically, it has been recently shown (by our and other groups) that the ENS interacts with multiple systems and has been implicated in the onset and progression of many diseases that not only affect the gastrointestinal tract but also the CNS (5). Due to the intricacy of its network and close proximity with many other tissues, studying the nervous system of the gut is challenging and requires expertise from distinct fields. Our group and collaborators have all necessary tools and skills to thoroughly dissect the cellular and molecular pathways that underlie the ENS crosstalk with other systems in

	various conditions. Given the similarities in composition, organisation and function between the ENS and the CNS, investigating the role of the ENS in homeostasis and disease would allow scientists to take the complexity of the gut to study other organs. We expect to unite different fields to unravel many other biological questions raised in this project and contribute for the consolidation of enteric neuroscience. Relevance to the patient: Increasing evidence shows that the ENS might be the "entrance door" for several pathologies, including those affecting brain homeostasis. In neurodegenerative diseases, for instance, ENS phenotype and gastrointestinal malfunction have been shown to precede brain and/or motor symptoms by several years (5). Moreover, the ENS is likely to contribute to carcinogenesis, as both neo-neurogenesis and perineural invasion are unfavourable factors for CRC patients, which indicates that their survival rate is negatively affected by the higher nerve density in the tumour area (i.e. neo-neurogenesis) and invasion of tumour cells throughout nerve fibres (i.e. perineural invasion) (6). With confidence, it is conceivable to suggest that the participation of the ENS in diseases that affect the gastrointestinal tract and systems beyond it (i.e., gut-brain axis) deserves more attention. We aim to study whether the ENS functions as a key player in the maintenance of the fitness of the intestinal microenvironment, and its crosstalk with the brain. Furthermore, we consider the ENS as a potential target for therapies in diseases that affect the homeostasis of the gastrointestinal tract in humans. Our prospect is thus to unravel the role of ENS behaviour in homeostasis and under circumstances that disturb the equilibrium of the healthy gastrointestinal tract. Therefore, we will include relevant animal and in vitro models to mimic human conditions, (e.g., cancer and helminthic infections), that impair intestinal function. By using advanced technology to study the ENS and powerful insights from experts in gastroenterology, we intend to identify novel cellular and molecular mechanisms, and biomarkers (e.g., EV-derived small molecules, non-coding miRNAs) that translate the onset of diseases progression and/or (response to) treatment.
Onderbouwing wetenschappelijk en maatschappelijk belang	Het belang is voldoende uitgewerkt en onderbouwd.

Wetenschappelijke kwaliteit Kwaliteit aanvrager/ onderzoeksgroep en onderzoek	Citaat C7 uit het DEC advies: Voor zover de 5.1 lid2h kan beoordelen zijn de kennis en kunde van de onderzoeksgroep adequaat gezien de jarenlange ervaring met proefdiermodellen voor het bestuderen van het enterisch zenuwstelsel, wetenschappelijke output, de verworven interne- en externe financiering alsmede de aandacht voor de drie V's onder meer geïllustreerd aan de hand van publicaties in tijdschriften als Nature Reviews Gastroenterology & Hepatology, American Journal of Physiology Gastrointestinal and Liver Physiology, J Neurogastroenterology and Motility en Glia. Het Secretariaat heeft geen reden hieraan te twijfelen.

3V's

Vervanging	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: If possible, we will perform the experiments using established in vitro cell lines. Only when these techniques are not optimal to investigate our research questions, or if they do not work, will we use the primary cell cultures to investigate our hypothesis. For example, no ENS cell lines are available to study our research questions, therefore primary cell cultures need to be used. We aim to validate our data using cell cultures derived from human intestinal tissue specimens. However, compared to the high murine cell yield and tissue availability, a limited number of human tissue samples is available and the cell yield is also narrow, thereby limiting the possibility to adequately and preferentially use human samples. Furthermore, human samples cannot be easily manipulated to have the same potential as our mouse lines.

	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models to study CRC. In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied in vivo. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established in vitro culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis in vivo. We are also taking advantage of recently established collaborations (e.g. in the gastroenterology clinic (Maastricht University)) to obtain human intestinal specimens and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study cancer development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.

	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Citaat: To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models, for instance the ones described in this document (such as H. poly infection models). In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied in vivo. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established in vitro culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis in vivo. We are also taking advantage of recently established collaborations in the gastroenterology clinic (Maastricht University) to obtain human intestinal specimens (IBDs and IBS) and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study disease development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Verminderen	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: We will limit the number of animals by isolating different cell types from the intestinal tract of the same mouse and by using animals that come from breedings carried out for the procedures in appendix 2/3, but cannot be used there.

	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: Before embarking on procedures in animal research, we will collect evidence form in vitro/in silico procedures if possible to determine whether a candidate gene or molecule also provide insights on in vivo pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This can also be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals (mostly included in the previous PL (AVD 5.1 lid2h), so not included in the current strategy). This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.

	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Citaat: Before embarking on any procedures in animal research, we are collecting as many evidence as it is necessary to determine whether a candidate gene or molecule also provide insights on in vivo pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This is also can be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals. This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.

Verfijnen	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: We do not expect to culture cells from any animals that display harmful phenotypes. Our experimental in vitro approach will allow us to gather very specific insights prior to doing any in vivo procedures.
	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no phenotype and/or useful results, experiments that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and/or well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached.
	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Zie bijlage 3.4.3.2. Citaat: Discomfort level in inflammatory, H. poly models might be mild to moderate (DSS). In the case of severe discomfort, mice will be immediately sacrificed.

Hergebruik	Er is geen sprake van hergebruik van dieren.

Naam proef	Worden de dieren gedood?	Doden volgens richtlijn?
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres	Ja	volgens de richtlijn.
3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer	Ja	volgens de richtlijn.
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases	Ja	volgens de richtlijn.

Naam proef		
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres	HEP: Worden niet verwacht	
Muizen (Mus musculus)	Ongerief: 100,0% Licht	

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer	HEP: 10-15%	Citaat: • All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist. • Any animal will be immediately euthanised if it exhibits signs of suffering that is not transient or treatable, and compromises its normal behaviour. • Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe discomfort and >20% for severe discomfort) that persist for 24 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision. • Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled. • Chemical CRC models: animals undergo euthanasia about 100 days after the beginning of the procedure. Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 2), "Wasting Syndrome" (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), or opportunist infections, the animals will be immediately euthanised. • Radio- and Chemotherapies: adverse effects such as inflammation and skin conditions such as loss of coat pigmentation may appear. Animals can undergo moderate to severe adverse effects and will be culled if signs of severe discomfort persist such as facial expression score of 3, "Wasting Syndrome" (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), coat/skin condition 3 for at least 3 consecutive days, chronic diarrhea or opportunist infections.

Muizen (Mus musculus)	Ongerief: 15,0% Ernstig 35,0% Matig 50,0% Licht	Omdat er een go-no go moment als voorwaarde in de beschikking wordt opgenomen zal ernstig ongerief voorkomen worden.
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases	HEP: 10-15%	Citaat: All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persists for longer than 24 hours. • Any animal will be immediately euthanised if it exhibit signs of suffering that is not transient or treatable and compromises its normal behaviour. • Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe disomfort and >10% for severe discomfort) that persist for 48 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision. • Genetically altered mice used in this appendix are not expected to show any deleterious phenotype, but in case they do, they will be immediately killed. • Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled. • Inflammation models: Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 3), "Wasting Syndrome" (body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), chronic diarrhea or opportunist infections, the animals will be immediately euthanised. • In the case severe discomfort occur in multiple mice during the experimental procedures described abobe, the experiments will be ended and the animals euthanised. • H. poly - Some weight loss is expected but mice

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bij bijlage 2 is dit 24 uur, is er een reden dat dit hier anders is?

		carrying other mutations may show more severe responses. Any mice showing weight loss of 15% of starting weight or diarrhoea, bloody stools, rectal prolapse, abdominal discomfort and bloating for more than 24h will be killed.
Muizen (Mus musculus)	Ongerief: 35,0% Matig 65,0% Licht	

5 Samenvatting

5.2 lid1

In bijlage 2 van de dierproeven zullen dieren voordat ze een CT scan krijgen 8 uur worden gevast. 5.2 lid1

Het uitgebrachte advies van de DEC is tot stand gekomen op basis van een meerderheidsstandpunt. 1 lid nam het minderheidsstandpunt in. De overwegingen hiervoor waren het aantal dieren en het gebruik van genetisch gemodificeerde muizen.

Daarnaast is er in de DEC gediscussieerd de haalbaarheid in de gestelde tijdsduur en over het ernstige ongerief dat dieren kunnen ondergaan. De DEC stelt voor om de volgende voorwaarde op te nemen in de beschikking:

Dat de dieren niet meer dan matig ongerief zullen ondervinden. De onderzoekers geven aan dat uitkomsten van pilotstudies op een lopende vergunning zorgen voor een go no go voor het deel van de experimenten die hier worden aangevraagd. Daarbij is een no-go van toepassing wanneer uit de pilot-experimenten blijkt dat ernstig ongerief niet vermeden kan worden.

5.2 lid1

Daarbij geeft de aanvrager in bijlage 2 van de dierproeven van het aangevraagde onderzoek zelf ook aan deze pilot als go-no go te hanteren. Ze schatten zelf het ongerief nog wel in op ernstig. Het Secretariaat heeft gevraagd dit in de bijlage dierproeven aan te passen. Met het opnemen van de voorwaarde in de beschikking wordt ernstig ongerief bij de dieren voorkomen.

6 Voorstel besluit incl. voorstel geldigheidsduur van de vergunning

5.2 lid1

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volgens de DEC 16 uur

Nummer: 2 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 17:00:41
en de DEC?

Nummer: 3 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 17:30:11
ik zou ook iets zeggen over dat het onderzoek fundamenteel is en het onzeker is wat voor therapieën hieruit voort kunnen komen

Nummer: 4 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 17:01:00
over?

Nummer: 5 Auteur: 5.1 lid2e Onderwerp: Notitie Datum: 31-5-2022 17:01:45
ik doe altijd go/no-go maar misschien zijn er verschillende spelwijzen goed

5.2 lid1

Voorwaarden
In de lopende projectaanvraag AVD5.1 lid2h zal bij bijlage 2 van de dierproeven een pilot worden uitgevoerd waarbij wordt gekeken of ernstig ongerief bij de gegeven dosis chemo-en radiotherapie met de CRC modellen kan worden vermeden. Als hieruit blijkt dat ernstig ongerief niet vermeden kan worden dan geldt dit als een no-go moment en mag het deel in bijlage 2 van de dierproeven waarbij chemo-en radiotherapie worden gegeven van dit aangevraagde onderzoek niet uitgevoerd worden.

De ingangsdatum van de vergunning kan niet voor de verzenddatum van de beschikking zijn en zal indien van toepassing aangepast worden. Dit is ook het geval bij een voorgenomen besluit.

7 Concept beschikking voor akkoord CCD

Advies aan CCD

Datum 31 mei 2022
Betreft Advies Secretariaat over Aanvraag projectvergunning Dierproeven AVD202215867

Instelling:	5.1 lid2e
Onderzoeker:	5.1 lid2h
Project:	The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease
Aanvraagnummer:	AVD202215867
Betreft:	Nieuwe aanvraag
Categorieën:	Fundamenteel onderzoek

1 Inzicht in aanvraag en de eventuele knelpunten en risico's

Proces	Er zijn geen vragen gesteld aan de DEC. Aan de aanvrager is gevraagd over de NTS: Uw aanvraag draait om het enterisch zenuwstelsel. Kunt u in de NTS helder uitleggen wat dit is? Daarnaast bevat de NTS veel lastige termen die niet begrijpelijk zullen zijn voor een leek. Kunt u de NTS beter navolgbaar maken? U benoemt bij vervanging het 5.1 lid2h De NTS dient echter anoniem te zijn. Kunt u het 5.1 lid2h uit de NTS halen? Kunt u mild ongerief veranderen naar licht ongerief? Over bijlage 2 van de dierproeven: Kunt u in bijlage 2 van de dierproeven bij C aanvullen hoe lang de dieren geen voedsel krijgen voordat ze een CT scan krijgen? Wanneer in bijlage 2 van de dierproeven uit de pilot waarin de dosis chemo-en radiotherapie wordt onderzocht blijkt dat ernstig ongerief wordt voorkomen dan zal dit een 'go' moment zijn. Hierdoor zullen de dieren in het aangevraagde onderzoek geen ernstig ongerief ondervinden. Kunt u bij bijlage 2 van de dierproeven bij F de classificatie van het cumulatieve ongerief in procenten aanpassen?

Naam proef	Diersoort	Stam	Aantal dieren	Herkomst
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres				
	Muizen (Mus musculus)		1.322	Dieren die voor onderzoek gefokt zijn
3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer				
	Muizen (Mus musculus)		2.118	Dieren die voor onderzoek gefokt zijn
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases				
	Muizen (Mus musculus)		2.385	Dieren die voor onderzoek gefokt zijn

Huisvesting en verzorging anders dan Bijlage III Richtlijn

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

Citaat: If mice have to undergo CT imaging, they need to be food deprived before performing the CT scan, so that no fecal pellets are present in the colon during imaging. Food will be given immediately when animals have undergone their CT scan.

Gebruik van mannelijke en vrouwelijke dieren

3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

Locatie uitvoering experimenten	- Alle proeven vinden plaats in een instelling van een vergunninghouder. - Er zijn geen problemen bekend met de vergunninghouder.

2 DEC advies

DEC-advies	Citaat vraag DEC aan de aanvrager (vraag 38): Waarom worden dieren aangevraagd voor experimenten die ook omschreven zijn binnen vorig PV, welke nog loopt? Worden de experimenten beschreven in vorig PV niet meer uitgevoerd, hoewel er nog een jaar looptijd is?

	Citaat antwoord aanvrager (vraag 38): We hebben hier enkel dezelfde diermodellen aangevraagd als in ons vorige PV als er nieuwe outcome parameters zijn toegevoegd die niet in onze huidige PV staan of we hebben voor dezelfde uitcome parameters nieuwe diermodellen aangevraagd. Een uitzondering hierop zijn de dieren voor therapie. Deze experimenten zijn niet meer haalbaar in de lopende PV en zijn dus verplaatst naar deze aanvraag. Citaat vraag DEC aan de aanvrager (vraag 47): 10 CTs leidt tot een cumulatieve bestralingsdosis (whole body) van minimaal 3 Gy. Welk effect heeft dit op de uitleesparameters van de desbetreffende experimenten? En op het ongerief? Wat is de stralingsgevoeligheid van de muizenstammen die u wilt gebruiken? Citaat antwoord aanvrager (vraag 47): Ongerief zal zoals beschreven, moderate-to-severe zijn. De pilootexperimenten die onder de huidige PV beschreven staan, zullen echter gebruikt worden om het effect van de therapie te bestuderen en het ongerief zoveel mogelijk te beperken. Om de stralingsgevoeligheid, de dosis en frequentie te bepalen zullen we samenwerken met andere onderzoekers die vergelijkbare experimenten eerder hebben uitgevoerd. Citaat vraag DEC aan de aanvrager (vraag 53): U geeft aan dat bestraling en behandeling met chemotherapie van de muizen tot “moderate (to severe)’ ongerief kan leiden. Verwacht u hier maximaal ernstig of maximaal matig ongerief? Citaat antwoord aanvrager (vraag 53): We hebben hier op dit moment nog maximaal ernstig ongerief beschreven. We gaan echter met de pilootstudies onder onze huidige PV deze procedures optimaliseren en daarmee verwachten we, maar zijn we niet geheel zeker dat, het ongerief terug te kunnen brengen naar matig ongerief. Citaat vraag DEC aan de aanvrager (vraag 4, ronde 2): De 5.1 lid2h kan geen ethische inschatting maken over het therapie gedeelte (ongerief, humane eindpunten) aangezien wordt aangegeven dat dit afhankelijk is van de uitkomst van pilootstudies die nog dienen te gebeuren op het huidig lopende PV (en daarom nu als maximaal ernstig wordt ingeschat). Echter op vraag 38 geeft u als antwoord dat alle therapie studies van huidig lopend PV niet meer zullen uitgevoerd worden en daarom zijn meegenomen in dit PV. Dit lijkt dus een belangrijke tegenstrijdigheid te zijn. Bovendien is het feit dat dieren ernstig ongerief kunnen ondervinden een belangrijk ethisch dilemma in deze aanvraag. Daardoor kan de

	5.1 lid2h over dit gedeelte nu geen beslissing nemen. We stellen daarom voor om OF deze pilootstudies mee te nemen in huidige aanvraag met een duidelijke go/no-go beslissing naar de eigenlijke therapie studies (inclusief zo veel mogelijke beperking van het ongerief) OF de therapie-studies uit de aanvraag te halen. Citaat antwoord aanvrager (vraag 4, ronde 2): Zoals correct opgemerkt zullen enkel de pilootstudies, maar niet de officiële therapiestudies uitgevoerd worden op het lopende PV. Deze huidige PV loopt namelijk nog 1,5 jaar, waarin wij verwachten deze pilootstudies uit te voeren. Daarom zijn deze pilootstudies dus niet meer opgenomen in deze nieuwe PV aanvraag. De therapiestudies zelf krijgen we niet meer uitgevoerd in de komende 1,5 jaar, waardoor deze wel opgenomen zijn in deze nieuwe PV. Om ervoor te zorgen dat er een goede ethische overweging gemaakt kan worden hebben we nu een duidelijker go/no-go moment toegevoegd (appendix 2, p.5). De pilootstudies uit de lopende PV zullen als go/no-go moment gelden voor de therapiestudies in deze PV. In het geval het niet mogelijk blijkt (op basis van de pilootstudies) om de therapiestudies met matig ongerief uit te voeren, zullen deze experimenten vervallen (max 432 dieren). Het ongerief zal hierdoor zoveel mogelijk beperkt worden en wordt geschat op moderate. Citaat C10: Een deel van de dieren zal maximaal 16 uur zonder voedsel gezet worden, zodat er geen voedsel in de darmen zit bij het uitvoeren van de CT-scans. De 5.1 lid2h is er verder van verzekerd dat voldaan wordt aan huisvesting en verzorging volgens de richtlijn op basis van de daartoe strekkende verklaring (in duplo) van zowel de vertegenwoordiger van de vergunninghouder, als de aanvrager onder respectievelijk punt 6 der ondertekening van de aanvraag en punt F in de bijlagen. Citaat C11: De 5.1 lid2h acht het ongerief grotendeels realistisch ingeschat. In het voorstel wordt aangegeven dat in totaal 68% van de dieren licht, 23% matig en 9% ernstig ongerief zal kunnen ervaren. De 5.1 lid2h vindt dat ernstig ongerief binnen deze aanvraag niet gerechtvaardigd is. Uit de antwoorden van de onderzoekers (vraag 4, tweede ronde) is namelijk gebleken dat er op een lopende vergunning pilotstudies uitgevoerd zullen worden waarbij de optimale omstandigheden voor de experimenten met potentieel ernstig ongerief bepaald worden. Wanneer de uitkomst van de pilotstudies is dat er ernstig ongerief zal optreden, is dit aangemerkt als een 'no-go' voor de betreffende vervolg-experimenten binnen deze aanvraag. Ethische afweging van de DEC: Citaat:

	1. Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan? 2. -Waarden die voor de proefdieren in het geding zijn: substantieel nadeel -Waarden die voor onderzoekers bevorderd worden: reëel voordeel. -Waarden die voor de medische wetenschap bevorderd worden: reëel voordeel. De 5.1 lid2h is van mening dat de belangen van onderzoekers/wetenschappelijke instituten, binnen het project 'The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease' zwaarder wegen dan de belangen/waarden van de proefdieren/dierbeschermingsorganisaties, mits ernstig ongerief wordt voorkomen. Voor de betrokken proefdieren leiden de beschreven proeven tot de dood na, in het voorstel aangegeven licht ongerief voor 68% van de dieren, matig ongerief voor 23% van de dieren en ernstig ongerief voor 9% van de dieren. De 5.1 lid2h is echter van mening dat ernstig ongerief voorkomen dient te worden, omdat er een go-no go is ingebouwd door de onderzoekers tussen deze aanvraag en een reeds lopende vergunning. Wanneer uit de pilotstudies onder de lopende vergunning blijkt dat ernstig ongerief niet vermeden kan worden, zullen de betreffende experimenten in de huidige aanvraag geen doorgang vinden (zie antwoord op vraag 4 uit de tweede ronde vragen). De dieren worden door de experimenten in hun welzijn geschaad. De integriteit van de dieren zal worden aangetast door de experimentele handelingen (injecties (o.a. voor het induceren van genetische veranderingen en het toedienen van chemische middelen), inductie van darmkanker (via injecties, of via colonoscopie), CT-scans (met bijbehorende voedseldeprivatie), bestraling, chemotherapie, verschillende manieren voor het induceren van darminflammatie (worminfectie, dextraan natriumsulfaat (DSS)), laparotomie, het veranderen van de darmflora (antibioticumkuur gevolgd door fecaal transplantaat, vasten) en het leven met de gevolgen daarvan gedurende de proeven en de opoffering aan het eind daarvan. Indien de doelstellingen bereikt worden, zal dit project echter leiden tot meer inzicht in de communicatie tussen het enterische zenuwstelsel en andere celtypen in homeostase of onder pathologische omstandigheden

zoals inflammatie, kanker of veranderingen in de microbiota. Daardoor zullen aandoeningen aan de darm mogelijk beter begrepen worden en worden deuren geopend naar nieuwe interventies.

Het is aannemelijk dat de doelstelling behaald zal worden. De onderzoekers zullen zoveel mogelijk trachten het lijden van de dieren te beperken.

3. De 5.1 lid2h beantwoordt de centrale morele vraag 'Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan?' bevestigend.

De 5.1 lid2h onderschrijft de integriteit en intrinsieke waarde van het dier en heeft oog voor het te ondergane ongerief van de proefdieren. Naar haar mening weegt het reële belang van dit project, en meer specifiek de belangen van de onderzoekers en het betreffende onderzoeksgebied zwaarder dan de voorgestelde schending van integriteit, het te berokkenen ongerief en opoffering, mits ernstig ongerief wordt vermeden.

De 5.1 lid2h is van mening dat de voorgestelde experimentele opzet en de uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en dat de gekozen strategie en de voorgestelde experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het programma. De onderzoekers beschikken over de benodigde kennis en technische expertise, zoals duidelijk uit hun voorstel blijkt. Er is geen sprake van duplicatie.

In de gekozen strategie wordt op bevredigende wijze tegemoetgekomen aan de vereisten van vervanging, vermindering en verfijning. De 5.1 lid2h is ervan overtuigd dat de aanvrager voldoende maatregelen treft om zowel het ongerief van de dieren als het aantal benodigde dieren tot een minimum te beperken. Er zijn voldoende go/no-go momenten voorzien om onnodige dierproeven te vermijden. De 5.1 lid2h is ervan overtuigd dat er geen alternatieven zijn, waardoor deze dierproef met minder ongerief of met minder, dan wel zonder levende dieren zou kunnen worden uitgevoerd. Wel is zij van mening dat ernstig ongerief vermeden kan en moet worden.

Op grond van deze overwegingen beschouwt de 5.1 lid2h de voorgestelde dierproeven in het projectvoorstel "The crosstalk between the enteric

	nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" als ethisch gerechtvaardigd, mits ernstig ongerief wordt vermeden. Derhalve voorziet de 5.1 lid2h het projectvoorstel "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" van een positief advies onder voorwaarde dat ernstig ongerief voor de dieren vermeden zal worden. De DEC heeft extern advies ingewonnen bij - de aanvrager is om aanvullingen gevraagd De DEC heeft een groot aantal vragen gesteld, opgenomen in 2 bijlagen van het DEC advies. Het DEC advies is Verlenen onder voorwaarden De onderzoekers geven aan dat uitkomsten van pilotstudies op een lopende vergunning zorgen voor een go-no go voor het deel van de experimenten die hier worden aangevraagd. Daarbij is een no-go van toepassing wanneer uit de pilot-experimenten blijkt dat ernstig ongerief niet vermeden kan worden. Het uitgebrachte advies is niet gebaseerd op consensus. Citaat: Het uitgebrachte 5.1 lid2h advies is tot stand gekomen op basis van een meerderheidsstandpunt. Een DEC-lid nam een minderheidsstandpunt in. De overwegingen hiervoor waren: (1) het grote aantal muizen staat nauwelijks in verhouding tot de praktische baten van het onderzoek; aangezien het fundamenteel onderzoek betreft is het onzeker of hier nieuwe therapieën uit voort gaan komen; (2) het gebruik van genetisch gemodificeerde muizen. De volgende dilemma's zijn gesignaleerd door de DEC: Citaat: Het uitgebrachte 5.1 lid2h advies is tot stand gekomen op basis van een meerderheidsstandpunt. Een DEC-lid nam een minderheidsstandpunt in. De overwegingen hiervoor waren: (1) het grote aantal muizen staat nauwelijks in verhouding tot de praktische baten van het onderzoek; aangezien het fundamenteel onderzoek betreft is het onzeker of hier nieuwe therapieën uit voort gaan komen; (2) het gebruik van genetisch gemodificeerde muizen.

3 Kwaliteit DEC advies

Kwaliteit DEC-advies	
Het DEC advies is helder en navolgbaar. Bij de beantwoording van de beoordelingsvragen verstrekt u een heldere onderbouwing. De CCD had bij vraag C10 graag de mening van de DEC gezien. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. Het valt op dat u zeer veel vragen hebt gesteld. Hoewel dit de kwaliteit van de aanvraag verbetert, wekt het ook de suggestie van meeschrijven, wat onwenselijk is. U heeft in uw advies een voorwaarde voorgesteld. Het is de CCD duidelijk waarom u de voorgestelde voorwaarde wilt stellen. De CCD stelt het op prijs dat goed navolgbaar in uw advies is opgenomen dat het advies is gebaseerd op een meerderheidsstandpunt, en waarop het minderheidsstandpunt op gebaseerd is. Ook stelt de CCD het op prijs dat bij E3 goed navolgbaar is weergegeven waar discussie over is geweest tijdens de bespreking van deze aanvraag.	

4 Inhoudelijke beoordeling

Doelstelling Doelstelling	Citaat: The ultimate goal of this project is to get a better understanding of the molecular pathways that orchestrate the organisation and function of the ENS, and its integration with other tissues (e.g., brain) and systems (e.g., immune, vascular and epithelial systems) during physiological challenges (e.g. microbiota) and pathological challenges (e.g. gastrointestinal diseases like cancer, worm infection). This will bring us closer to understand how the ENS participates in the onset and progression of diseases that affect the gastrointestinal tract, and therefore will eventually contribute to the development of new targets and therapies to tackle gastrointestinal disorders. For this purpose, we aim to develop a holistic and advanced approach to study the influence of the intestinal environment on ENS transcriptome, morphology and physiology, both in health and disease, for which we will use well-established murine models and procedures.
Wetenschappelijk en maatschappelijk belang	Citaat: Relevance for research: Research interest focusing on the gut has increased in recent years because of the clinical and biological relevance of this organ in several diseases. More specifically, it has been recently shown (by our and other groups) that the ENS interacts with multiple systems and has been implicated in the onset and progression of many diseases that not only affect the gastrointestinal tract but also the CNS (5). Due to the intricacy of its network and close proximity with many other tissues, studying the nervous system of the gut is challenging and requires expertise from distinct fields. Our group and collaborators have all necessary tools and skills to thoroughly dissect the cellular and molecular pathways that underlie the ENS crosstalk with other systems in

	various conditions. Given the similarities in composition, organisation and function between the ENS and the CNS, investigating the role of the ENS in homeostasis and disease would allow scientists to take the complexity of the gut to study other organs. We expect to unite different fields to unravel many other biological questions raised in this project and contribute for the consolidation of enteric neuroscience. Relevance to the patient: Increasing evidence shows that the ENS might be the "entrance door" for several pathologies, including those affecting brain homeostasis. In neurodegenerative diseases, for instance, ENS phenotype and gastrointestinal malfunction have been shown to precede brain and/or motor symptoms by several years (5). Moreover, the ENS is likely to contribute to carcinogenesis, as both neo-neurogenesis and perineural invasion are unfavourable factors for CRC patients, which indicates that their survival rate is negatively affected by the higher nerve density in the tumour area (i.e. neo-neurogenesis) and invasion of tumour cells throughout nerve fibres (i.e. perineural invasion) (6). With confidence, it is conceivable to suggest that the participation of the ENS in diseases that affect the gastrointestinal tract and systems beyond it (i.e., gut-brain axis) deserves more attention. We aim to study whether the ENS functions as a key player in the maintenance of the fitness of the intestinal microenvironment, and its crosstalk with the brain. Furthermore, we consider the ENS as a potential target for therapies in diseases that affect the homeostasis of the gastrointestinal tract in humans. Our prospect is thus to unravel the role of ENS behaviour in homeostasis and under circumstances that disturb the equilibrium of the healthy gastrointestinal tract. Therefore, we will include relevant animal and in vitro models to mimic human conditions, (e.g., cancer and helminthic infections), that impair intestinal function. By using advanced technology to study the ENS and powerful insights from experts in gastroenterology, we intend to identify novel cellular and molecular mechanisms, and biomarkers (e.g., EV-derived small molecules, non-coding miRNAs) that translate the onset of diseases progression and/or (response to) treatment.
Onderbouwing wetenschappelijk en maatschappelijk belang	Het belang is voldoende uitgewerkt en onderbouwd.

Wetenschappelijke kwaliteit Kwaliteit aanvrager/ onderzoeksgroep en onderzoek	Citaat C7 uit het DEC advies: Voor zover de 5.1 lid2h kan beoordelen zijn de kennis en kunde van de onderzoeksgroep adequaat gezien de jarenlange ervaring met proefdiermodellen voor het bestuderen van het enterisch zenuwstelsel, wetenschappelijke output, de verworven interne- en externe financiering alsmede de aandacht voor de drie V's onder meer geïllustreerd aan de hand van publicaties in tijdschriften als Nature Reviews Gastroenterology & Hepatology, American Journal of Physiology Gastrointestinal and Liver Physiology, J Neurogastroenterology and Motility en Glia. Het Secretariaat heeft geen reden hieraan te twijfelen.

3V's

Vervanging	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: If possible, we will perform the experiments using established in vitro cell lines. Only when these techniques are not optimal to investigate our research questions, or if they do not work, will we use the primary cell cultures to investigate our hypothesis. For example, no ENS cell lines are available to study our research questions, therefore primary cell cultures need to be used. We aim to validate our data using cell cultures derived from human intestinal tissue specimens. However, compared to the high murine cell yield and tissue availability, a limited number of human tissue samples is available and the cell yield is also narrow, thereby limiting the possibility to adequately and preferentially use human samples. Furthermore, human samples cannot be easily manipulated to have the same potential as our mouse lines.

	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models to study CRC. In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied in vivo. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established in vitro culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis in vivo. We are also taking advantage of recently established collaborations (e.g. in the gastroenterology clinic (5.1 lid2h to obtain human intestinal specimens and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study cancer development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.

	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Citaat: To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models, for instance the ones described in this document (such as H. poly infection models). In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied in vivo. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established in vitro culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis in vivo. We are also taking advantage of recently established collaborations in the gastroenterology clinic (5.1 lid2h to obtain human intestinal specimens (IBDs and IBS) and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study disease development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Verminderen	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: We will limit the number of animals by isolating different cell types from the intestinal tract of the same mouse and by using animals that come from breedings carried out for the procedures in appendix 2/3, but cannot be used there.

	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: Before embarking on procedures in animal research, we will collect evidence form in vitro/in silico procedures if possible to determine whether a candidate gene or molecule also provide insights on in vivo pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This can also be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals (mostly included in the previous PL (AVD5.1 lid2h), so not included in the current strategy). This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.

	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Citaat: Before embarking on any procedures in animal research, we are collecting as many evidence as it is necessary to determine whether a candidate gene or molecule also provide insights on in vivo pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This is also can be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals. This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.

Verfijnen	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: We do not expect to culture cells from any animals that display harmful phenotypes. Our experimental in vitro approach will allow us to gather very specific insights prior to doing any in vivo procedures.
	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no phenotype and/or useful results, experiments that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and/or well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached.
	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Zie bijlage 3.4.3.2. Citaat: Discomfort level in inflammatory, H. poly models might be mild to moderate (DSS). In the case of severe discomfort, mice will be immediately sacrificed.

Hergebruik	Er is geen sprake van hergebruik van dieren.

Naam proef	Worden de dieren gedood?	Doden volgens richtlijn?
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres	Ja	volgens de richtlijn.
3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer	Ja	volgens de richtlijn.
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases	Ja	volgens de richtlijn.

Naam proef		
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres	HEP: Worden niet verwacht	
Muizen (Mus musculus)	Ongerief: 100,0% Licht	

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer	HEP: 10-15%	Citaat: • All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist. • Any animal will be immediately euthanised if it exhibits signs of suffering that is not transient or treatable, and compromises its normal behaviour. • Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe discomfort and >20% for severe discomfort) that persist for 24 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision. • Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled. • Chemical CRC models: animals undergo euthanasia about 100 days after the beginning of the procedure. Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 2), "Wasting Syndrome" (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), or opportunist infections, the animals will be immediately euthanised. • Radio- and Chemotherapies: adverse effects such as inflammation and skin conditions such as loss of coat pigmentation may appear. Animals can undergo moderate to severe adverse effects and will be culled if signs of severe discomfort persist such as facial expression score of 3, "Wasting Syndrome" (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), coat/skin condition 3 for at least 3 consecutive days, chronic diarrhea or opportunist infections.

Muizen (Mus musculus)	Ongerief: 15,0% Ernstig 35,0% Matig 50,0% Licht	Omdat er een go-no go moment als voorwaarde in de beschikking wordt opgenomen zal ernstig ongerief voorkomen worden.
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases	HEP: 10-15%	Citaat: All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persists for longer than 24 hours. • Any animal will be immediately euthanised if it exhibit signs of suffering that is not transient or treatable and compromises its normal behaviour. • Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe disomfort and >20% for severe discomfort) that persist for 48 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision. • Genetically altered mice used in this appendix are not expected to show any deleterious phenotype, but in case they do, they will be immediately killed. • Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled. • Inflammation models: Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 3), “Wasting Syndrome” (body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), chronic diarrhea or opportunist infections, the animals will be immediately euthanised. • In the case severe discomfort occur in multiple mice during the experimental procedures described abobe, the experiments will be ended and the animals euthanised. • H. poly - Some weight loss is expected but mice

		carrying other mutations may show more severe responses. Any mice showing weight loss of 15% of starting weight or diarrhoea, bloody stools, rectal prolapse, abdominal discomfort and bloating for more than 24h will be killed.
Muizen (Mus musculus)	Ongerief: 35,0% Matig 65,0% Licht	

5 Samenvatting

5.2 lid1 [redacted]

In bijlage 2 van de dierproeven zullen dieren voordat ze een CT scan krijgen 16 uur worden gevast. 5.2 lid1 [redacted].

Het uitgebrachte advies van de DEC is tot stand gekomen op basis van een meerderheidsstandpunt. 1 lid nam het minderheidsstandpunt in. De overwegingen hiervoor waren het aantal dieren tov het fundamentele onderzoek en de onzekere uitkomst of hier nieuwe therapieën uit voortkomen en het gebruik van genetisch gemodificeerde muizen.

Daarnaast is er in de DEC gediscussieerd over de haalbaarheid in de gestelde tijdsduur en over het ernstige ongerief dat dieren kunnen ondergaan. De DEC stelt voor om de volgende voorwaarde op te nemen in de beschikking:

Dat de dieren niet meer dan matig ongerief zullen ondervinden. De onderzoekers geven aan dat uitkomsten van pilotstudies op een lopende vergunning zorgen voor een go/no go voor het deel van de experimenten die hier worden aangevraagd. Daarbij is een no/go van toepassing wanneer uit de pilot-experimenten blijkt dat ernstig ongerief niet vermeden kan worden.

5.2 lid1 [redacted]

Daarbij geeft de aanvrager in bijlage 2 van de dierproeven van het aangevraagde onderzoek zelf ook aan deze pilot als go/no go te hanteren. Ze schatten zelf het ongerief nog wel in op ernstig. Het Secretariaat heeft gevraagd dit in de bijlage dierproeven aan te passen. Met het opnemen van de voorwaarde in de beschikking wordt ernstig ongerief bij de dieren voorkomen.

6 Voorstel besluit incl. voorstel geldigheidsduur van de vergunning

5.2 lid1

Voorwaarden
In de lopende projectaanvraag AVD5.1 lid2h zal bij bijlage 2 van de dierproeven een pilot worden uitgevoerd waarbij wordt gekeken of ernstig ongerief bij de gegeven dosis chemo-en radiotherapie met de CRC modellen kan worden vermeden. Als hieruit blijkt dat ernstig ongerief niet vermeden kan worden dan geldt dit als een no-go moment en mag het deel in bijlage 2 van de dierproeven waarbij chemo-en radiotherapie worden gegeven van dit aangevraagde onderzoek niet uitgevoerd worden.

De ingangsdatum van de vergunning kan niet voor de verzenddatum van de beschikking zijn en zal indien van toepassing aangepast worden. Dit is ook het geval bij een voorgenomen besluit.

7 Concept beschikking voor akkoord CCD

Van: info@zbo-ccd.nl
Verzonden: dinsdag 31 mei 2022 17:48
Aan: 5.1 lid2e ;5.1 lid2h
CC: 5.1 lid2e ;5.1 lid2h
Onderwerp: Aanhouden AVD5.1 lid2h202215867

Categorieën: 5.1 lid2e

Geachte dr. 5.1 lid2e

Op 17-02-2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease " met aanvraagnummer AVD5.1 lid2h202215867. 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
Uw aanvraag draait om het enterisch zenuwstelsel. Kunt u in de NTS helder uitleggen wat dit is?

Daarnaast bevat de NTS veel lastige termen die niet begrijpelijk zullen zijn voor een leek. Kunt u de NTS beter navolgbaar maken?

U benoemt bij vervanging het5.1 lid2h De NTS dient echter anoniem te zijn. Kunt u het5.1 lid2h uit de NTS halen?

Kunt u mild ongerief veranderen naar licht ongerief?

Onduidelijkheden
Kunt u in bijlage 2 van de dierproeven bij C aanvullen hoe lang de dieren geen voedsel krijgen voordat ze een CT scan krijgen?

Wanneer in bijlage 2 van de dierproeven uit de pilot waarin de dosis chemo-en radiotherapie wordt onderzocht blijkt dat ernstig ongerief wordt voorkomen dan zal dit een 'go' moment zijn. Hierdoor zullen de dieren in het aangevraagde onderzoek geen ernstig ongerief ondervinden. Kunt u bij bijlage 2 van de dierproeven bij F de classificatie van het cumulatieve ongerief in procenten aanpassen?

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

Opsturen binnen veertien dagen
Stuur de ontbrekende informatie binnen veertien dagen na de datum van dit bericht op. U kunt dit aanleveren via NetFTP.

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.

Mocht u vragen hebben, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

5.1 lid2e

www.centralecommissiedierproeven.nl

.....
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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'.	5.1 lid2h
1.2	Provide the name of the licenced establishment.	5.1 lid2h
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: Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

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.

Using the animal lines (table 1), models and procedures specified in this appendix, we aim to address RQ2 of our PL and investigate/ unravel the interaction of ENS cells (e.g., mature EGCs, neurons and enteric progenitor cells) with other intestinal cells (e.g., immune, vascular, epithelial, stromal cells), and extra intestinal tissues (e.g. microbiota) in health and colorectal cancer (CRC) *in vivo*. Hereby, we will explore the interaction between the ENS and CRC and the potential underlying mechanisms.
In addition, we will isolate tumors and adjacent normal epithelium, to generate intestinal (tumor) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact intra- and extracellular mechanism by which the ENS affects the epithelial and tumor cells. In case of success in acquisition and analysis of data using these models (e.g. differences in cancer induction (tumor number/size/growth) between genetic models) (go/no-go), we will also study the potential impact of an impaired ENS (genetically/chemically-induced alteration) on current treatments of CRC (e.g. radio- and chemotherapy). We will use wild-type mice and mice with a labelled or altered ENS (see table below) containing a non-diseased gut (control) and/or a diseased gut (colorectal cancer) (**Figure 1**).

Mouse lines:

Table 1: Mouse models and potential examples of mouse lines that can be used*:

Mouse model	Potential examples
Wild-type mice	

Mice with fluorescently labelled ENS cells	Sox10.CreER^{T2}:R26^{tdTomato} Wnt1.Cre:R26^{tdTomato} Sox10.CreER^{T2}/Wnt1.Cre:R26-GCaMP6f
Mice with fluorescently labelled extracellular vesicles from ENS cells	CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre
Transgenic mice with altered ENS	NDRG4$^{fl/fl}$Wnt1.Cre, (enteric neural specific Ndrg4 knockdown) NSE-Noggin (more enteric neurons) Hand2$^{fl/+}$:Wnt1.Cre (less enteric neurons)
Lines designed to specifically modulate ENS activity	Sox10.CreER^{T2}/Wnt1.Cre:R26-hM4Di-DREADD

* The mouse lines depicted within this table are at present the best models to investigate our research question. However, we would like to point out that better models might appear in the future (e.g. more efficient labelling of alteration of cells/molecules of interest). Consequently, the lines given here represent potential examples, by which means we will be able to change to better models in case these will be designed and/or become available. However, the number of models will remain the same.

Several primary outcomes **(see figure 1 – orange box)** will be analysed by using one, or a combination of the animal models described above (table 1)**:**

- ENS structure / (epi) genetic signature (e.g., morphology, ENS transcriptome/epigenome)
- ENS function (e.g., ENS connectivity and network activity)
- ENS proliferation and survival (e.g., cell death, proliferation, senescence)
- Development/progression of CRC (e.g., track tumour burden)
- Intercellular communication (e.g., ENS X immune, X vascular, X microbiota, X epithelial interactions)
- Gut physiology (e.g., intestinal motility, barrier function, endocrine signalling)
- Vesicle trafficking (e.g., intracellular vs. extracellular vesicles)
- Microbiota analysis (e.g., composition and abundance, bacteria secretome)

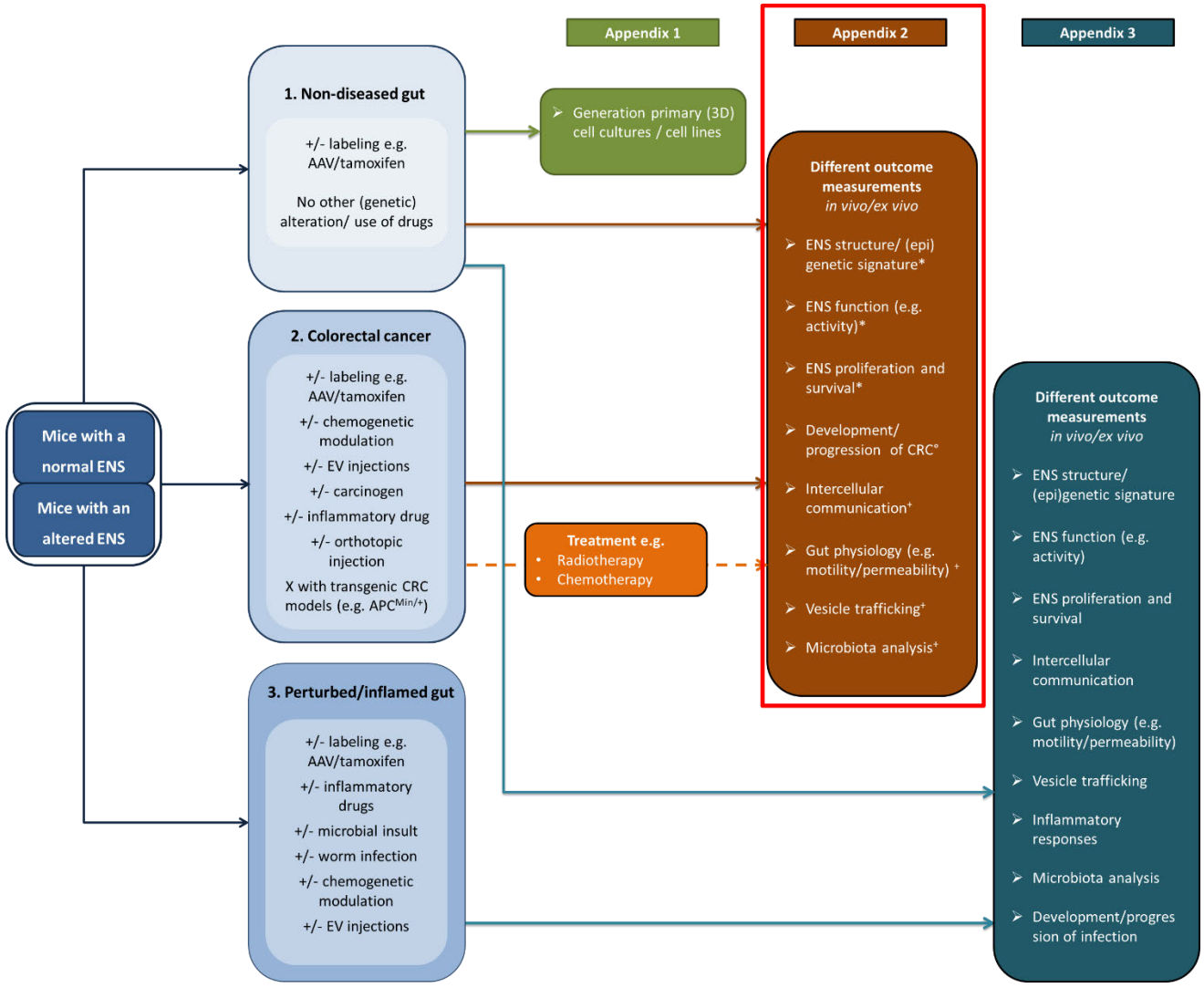

Figure 1. Outline depicting experimental models and outcome measures that will be assessed within each appendix. Red box delineates the purpose of this appendix.
With the experimental models and outcomes, we expect to further unravel mechanisms by which the ENS influences/responds to neighbouring environment in colorectal cancer by addressing the sub aims pointed below.

Aim 2: Investigate cellular and molecular mechanisms of interaction between ENS, intestinal (cancer) and other cells in a cancerous environment
Aim 2a: The role of EGCs in the development, progression, and treatment of CRC
Aim 2b: The role of enteric neurons in the development, progression, and treatment of CRC

This research will follow up on the research carried out under our previous project license 2017-026. Whereas animal lines, disease models and procedures partially overlap with PL2017-026, we have prevented as much overlap as possible. This is visualized in the table with all mouse numbers (word document – Total number of mice appendix 2) – where we omitted from the calculation of number of animals, the experiments/procedures that have already been performed. The data of these experiments demonstrate that the mouse lines and disease models are appropriate for studying the same outcome measures in other mouse lines and new outcome measures on both already in use and new mouse lines. The only overlap in animals that is present in both licenses is the therapy part as there is not enough time on the old project license to complete these experiments. This is due to the delay in establishing the correct mouse models and the Covid-19 pandemic.

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

To study how the ENS interacts with other cells, tissues and systems in health and CRC *in vivo* and *ex vivo*, we will take advantage of the well-established mouse lines described above (table 1). Animals can be subjected to CRC **(APC$^{Min/+}$, AOM, AOM/DSS, and/or orthotopic CRC model),** (epi)genetic editing (**AAVx-carrying genetic modifiers)**, and/or chemogenetic modulation **(DREADDs)** as described in the details below and in their respective programs of work. The same lines can be further examined after treatment for cancer **(radio- and chemotherapies)**.

Animal procedures for specific mouse models mentioned in table 1:

1. **Genetic editing of the ENS using viral vector transduction (AAVx.transgene) –** AAVs will be used to deliver genetic material to the ENS of mice to induce genetic alterations (e.g., deliver fluorescent reporters and/or silence, knockdown or overexpress genes) by e.g. tail vein injection, to analyse the response of the intestinal and extra-intestinal tissues.

2. **Chemogenetic modulation using designer receptors exclusively activated by designer drugs (DREADDs) -** AAVx vectors will be injected (e.g. tail vein injection) to target designer receptors exclusively activated by designer drugs (DREADD) variants to modulate ENS activity (e.g., activate or inhibit EGCs/enteric neurons). ENS structure, function and intercellular communication as well as gut physiology can be subsequently analysed**.** Similarly, DREADD receptors will be targeted to ENS cells using Sox10.CreERT2/Wnt1.Cre:R26-hM4Di-DREADD mice. Receptor activation will be achieved by providing clozapine-N-oxide (CNO) to the animals (via drinking water or ip injections).

Animal procedures for non-diseased or control vs diseased mice using the mouse models described in table 1:

1. **Control group** (e.g., untreated/sham mice and/or homozygous wildtype control with no expected deleterious phenotype)
    - For the mice with fluorescently labelled ENS cells and fluorescently labelled extracellular vesicles from ENS cells, this will be untreated/sham mice (so no cancer induction), but containing the labelled ENS cells or EVs.
    - For the transgenic mice with altered ENS and the lines designed to specifically modulate ENS activity, this will be (homozygous) wildtype controls that underwent the same procedures/colorectal cancer induction protocols as the transgenic mice.

2. **Colorectal Cancer (CRC) –** The following models will be used to trigger the onset of CRC.
    - *Genetic model:* APC$^{Min/+}$ mice spontaneously develop tumours predominantly in the small intestine within approximately 6 months. Based on previous observations with our NDRG4 mice, we expect the development of about 30 tumours in all models, resulting in mild discomfort. This model can be combined with the carcinogen AOM (injection) or the inflammatory drug DSS (in drinking water) (for more details, see the two model below) to induce tumours in the colon as well.
    - *Chemical carcinogen model*: Young adult mice (1-3 months) will be introduced to a well-established AOM carcinogen protocol (1 ip injection per week for 6 consecutive weeks). Tissues will be collected and analysed four months after the last AOM injection. Based on our previous observations by using this protocol, we expect the development of about 2-10 small colonic polyps in the models, causing mild discomfort.
    - *Chemical inflammatory/carcinogenic model:* Mice will be exposed to a combination of the inflammatory drug DSS (in drinking water) and the carcinogen AOM (ip injection), giving rise to colitis-associated cancer. Based on previous experience, we expect moderate discomfort and the appearance of about 10-40 colonic polyps, in the combined AOM/DSS model, but the development/growing of these polyps is faster than the AOM only model.
    - *Orthotopic CRC model:* Tumour cells (e.g. MC38 or CT26 cells) will be injected into the colon using a colonoscopy device when mice are under anaesthesia. A tumour will arise in every injection site (maximally three per mouse). Tumour metastasis and response to therapy (chemotherapy/radiotherapy) can be assessed in this model by CT imaging and evaluation after killing. Moderate to severe discomfort is expected.

      ***To evaluate the contribution of the ENS to the onset and progression of the disease, mice may also be killed prior to the appearance of polyps. However, CT scans can also be used for this purpose.**

      *** All CRC models used in this study do not have metastatic potential, so the mice will only develop tumours within the colon or small intestine, depending on the model used and the injection site.**

All four models have their specific characteristics and (dis)advantages. However, the chemical AOM (CRC model) and AOM/DSS (colitis-associated CRC model) model are the preferred models, because of the faster and more efficient CRC development and will therefore be used to study most of the outcome measures. Nevertheless, we will apply the genetic and orthotopic models to answer specific questions like labelled cell tracking (therapy experiments) and specific insights in human-specific CRC pathogenesis (when AOM or AOM/DSS models are not able to study certain outcome parameters with enough relevance for the patient). In addition, we will treat mice with CRC with either radiotherapy or chemotherapy, which can give us more insight into the mechanisms of treatment effects and the patients that would benefit from these treatments. Controls will receive sham therapy.

- Radiotherapy: Irradiation (non-lethal dose; therapeutic efficacy) onto the tumor tissue (via CT imaging): frequency: Max to be determined (range 5-10); Duration: max 30 min. Further details: Animals have to be food deprived before performing a CT scan (time depending on day-night rhythm, max 16h and mostly in the light phase), so no fecal pellets remain in the colon during imaging. At different time points after CRC induction, mice will be anesthetized for micro-CT imaging. The target intestinal tumors will be delineated, where after a beam will be placed as such to cover the target and to ensure optimal sparing of organs at risk. Around the tumors, a 1-mm margin will be included to ensure also irradiation of microscopic disease spread. These procedures will take about 15 minutes in anesthetized animals. Upon obtaining the optimal treatment plan, the target will be irradiated. After the procedures, animals are allowed to recover. Moderate (to severe) discomfort is expected from these procedures.
- Chemotherapy: Administration of for example 5-FU + oxaliplatin (at present the most used treatment for CRC) or saline: i.p. injection, dose per injection to be determined, max 10 injections. Moderate (to severe) discomfort is expected from these procedures.
  Of Note: Pilot experiments for establishing appropriate doses of chemo- and radio-therapy are included and will be performed using our previous PL (AVD5.1 lid2h) to minimize the discomfort and will be a ***go/no-go moment****. By carrying out the pilot experiments, we will try to make sure that we are able to find the most efficient dose and minimize the discomfort to moderate. If the pilot studies do not give rise to changes in tumor growth or if the discomfort on the animals is too high and cannot be minimized to moderate on average, we will not continue with the therapy studies included in this PL. However, given that these pilot experiments have not yet been performed, we have now taken into account the worst-case scenario, wherein animals may experience severe discomfort.*

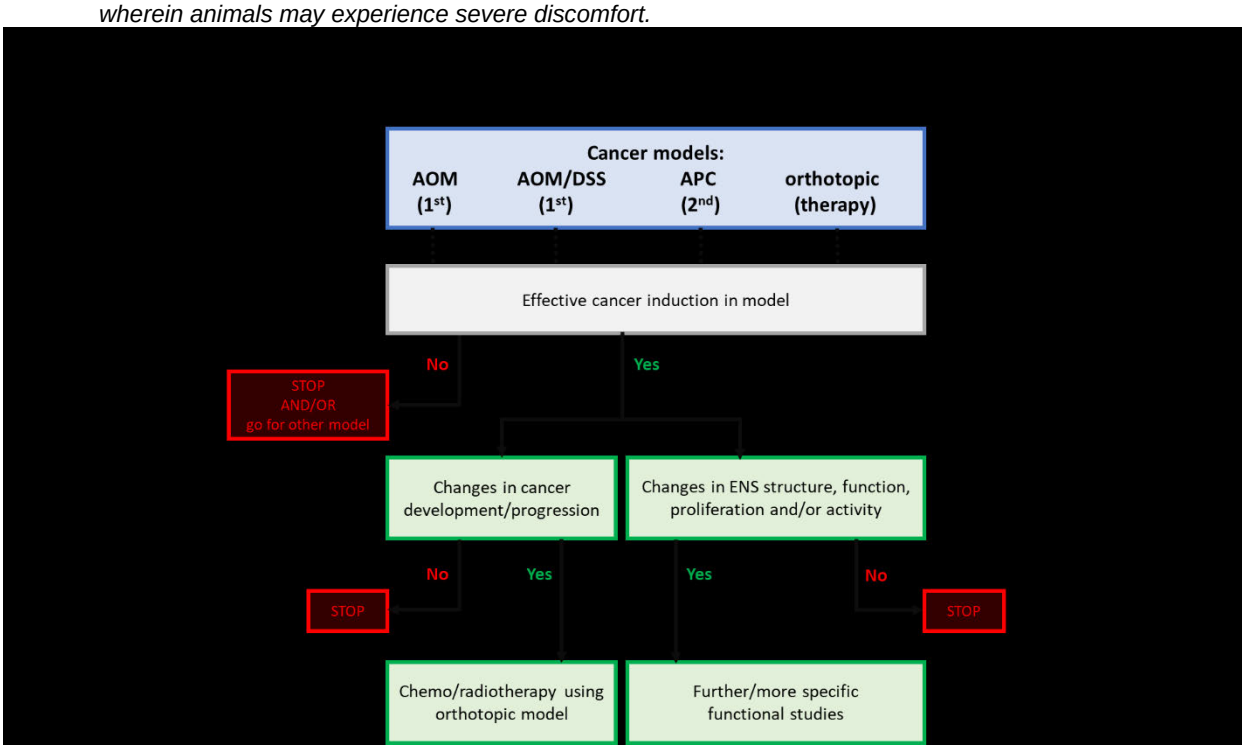

Figure 2. *Flowchart depicting the strategy and decision moments of the models (described above)/outcome measures (described below) in appendix 2.*

OUTCOME MEASUREMENTS THAT WILL BE ANALYSED:

As mentioned above, we will perform *in vivo* and/or *ex vivo* (post-mortem) analysis of the following outcome measures (the experimental methodologies that will be used are included as well). **Rationale: these outcome measures are included because they are ideally suited to reflect on the status and composition of the ENS, its modes of communication with, and effect on, surrounding tissues (e.g. tumour cells) and cellular systems (e.g. microbiota), and role in gut homeostasis (please also see background of Project Proposal).**

NB – Many of the outcome parameters can be analysed by using one group of animals (e.g., ENS structure, intercellular communication, gut physiology and microbiota analysis). Support on the group sizes and total number of animals can be found at section B. The subdivision of number of animals per procedure/model and the total number is depicted in detail within the following Word document: Total number of mice; table – appendix 2 – wherein we refer to the similar enumeration as used below in our explanation. As explained before, the mouse models in these tables are used as examples but can be substituted if better models become available.

- ***ENS structure/ (epi) genetic signature & ENS function & ENS proliferation and survival***

  Distinct imaging techniques will be used to study these parameters on ENS.

- We will examine the influence of CRC on the ENS composition, architecture, morphology and activity (**ex vivo)**:

a) ***Fate mapping experiments*** - we will use *Sox10.CreER^{T2}:R26td^{Tomato}*. Tamoxifen (i.p.) will be injected at/before the time of initiation of the CRC protocol to label Sox10 expressing EGCs with tdTomato reporter. **– 12 animals per group.**

b) ***Calcium imaging of ENS activity*** - We will examine the ENS activity *ex vivo* in different ways: i) [Ca2+]i-Fluo-4 imaging: Live recording of ENS activity will be performed using Fluo-4 Ca2+ -imaging in preparations of whole-mount mouse gut. ii) For the other Ca2+ imaging experiments we will use Sox10-CreERT2::R26-GCaMP6f mice (EGCs, tamoxifen injection in adult animals) and Wnt1.Cre:: R26-GCaMP6f mice (all enteric neurons and glia). **– 12 animals per group**

- We will examine the influence of CRC on the ENS regarding cell proliferation and survival

c) **For cell proliferation and survival:** Analysis of ENS proliferation at different stages during CRC development/progression. Animals will be given a thymidine analogue (e.g. BrdU, edU) to label cycling cells via i.p. injections and/or drinking water. *In vivo/ex vivo* labelling assays will be used to detect cell death, senescence, DNA damage. We will perform this experiment only if we observe any phenotype in organisation, composition and function of the ENS. **– 12 animals per group**

- We will investigate the molecular signature of ENS cells and/or extracellular components in gut homeostasis and CRC. We will use FACS to isolate ENS cells for qRT-PCR, bulk or single-cell RNA Sequencing

d) **Tissue isolation, digestion and FACS**
Intestinal preparations will be dissociated into single cell suspensions and subjected to FACS for isolation of ENS cells and/or other components and analysis by qRT-PCR, bulk and single cell RNA Sequencing. **- 9 animals per group**

- ***Development/progression of CRC***

  We will closely follow the onset and progression of tumours using the following techniques:

a) High resolution endoscopic monitoring - to analyse the effect of the treatment on tumour burden at different time points *in vivo*. **No culling, so performed in the mice that will be used at c.**

b) CT scans: This procedure will take about 15 minutes and will be done under anesthesia to prevent repositioning the animal. Contrast will be enhanced to visualize tumors using a protocol established in our lab and consists of an i.p. injection and a rectal injection of contrast agents. **No culling, so performed in the mice that will be used at c.**

c) All other outcomes will be analysed ex vivo (tumour number, size, histology, RNA expression, protein levels, cell isolation, neuron/EGC tracing, blood sampling etc.) **12 animals per group** to study histology and **6 animals per group** to study RNA/protein/cells.

d) This outcome measure will also be examined for the animals subjected to chemo- or radiotherapy. However, we need pilot studies with a smaller number of mice to first optimize the procedure before starting the experiment (go/no-go moment).

- ***Intercellular communication – 6 and 9 animals per group***

  We will investigate cell-to-cell communication between ENS cells and other intestinal and extra-intestinal systems (e.g. immune system, tumour cells, brain) by immunohistochemistry, qRT-PCR, FACS, western blotting, ELISA, etc. Mice will be euthanised and intestinal and extra-intestinal tissues will be collected for subsequent analysis. We will also isolate tumors and adjacent normal epithelium, to generate intestinal (tumor) organoids / ENS cells, which will be used in cell culture experiments to further identify the exact mechanism by which the ENS affects the epithelial and tumor cells.

  NB: The tissue collection will be performed separately only when it cannot be achieved together with other experiments, in order to minimise unnecessary culling and animal use.

- ***Gut physiology (intestinal motility & gut permeability)***

- To study intestinal motility ***in vivo*** we will implement different methods:

a) *Total intestinal transit time*: The protocol for the total gastrointestinal transit time will be performed as previously described[5]. Mice will be individually placed into cages devoid of bedding and fasted for an hour. Next, a dye will be administrated by oral gavage, and the animals returned to their individual cages. The total intestinal transit time will be calculated by the time from gavage until the extrusion of the first coloured pellet. A maximum period of observation will be 5 hours in all experiments and mice that fail to expel the red pellet within this time will be quantified as ending point at 5 hours. ***NB: No culling is needed, and mice can be used in other read-out parameters. The dye is cleared from the gastrointestinal tract 2 days after administration. Faeces will be collected and analysed for bacterial composition and stool quality.**

b) *Small intestinal transit:* As described for the total transit time, mice will be given dye via oral gavage. Mice will be euthanised, followed by removal of the small intestine. The total length of the small intestine and the distance that the dye has travelled will be measured to determine the small intestinal transit length. ***Faeces will be collected and analysed for bacterial composition and stool quality. Tissues will be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain).**

c) Colonic propulsion: Colonic propulsion will be evaluated by first lightly anesthetizing the animals with isoflurane. A small diameter glass bead will be inserted into the rectum, and the expulsion time of the glass bead will be recorded. The assay will be repeated twice every 100 min for a total of 3 bead insertions. ***NB: No culling is needed, and mice can be used in other read-outs.**

NB – experiments in a and c can be performed using the same group of animals – 12 animals per group.

- **In case of a phenotype in a, b and/or c -** To study the intestinal contractility and motility ***ex vivo*** using live video recording and spatiotemporal analyses of ENS-dependent motility. Experiments can be performed using tissue derived from animals used in b.

d) Segments from distinct parts of the gut of young adult mice will be removed as previously described. Segments will be carefully isolated, luminal contents emptied and placed loosely pinned onto an organ bath chamber continuously infused with Krebs solution and constantly supplied with 95% O2 and 5% CO2 at 37°C and the neurogenic intestinal motility recorded.

- To study gut permeability ***in vivo***:

e) *Measuring levels of plasma FITC:* - Intestinal permeability will be determined by measuring levels of plasma FITC after administration via oral gavage of FITC-conjugated dextran in PBS. Blood will be obtained after administration, and the concentration of fluorescein will be determined by spectrophotofluorometry. Mice will be euthanized during this experiment. ***Faeces can be collected and analysed for bacterial composition and stool quality. Tissues can be collected after euthanasia (e.g., SI, colon, immune cells compartments, brain). – 12 animals per group**

- ***Vesicle trafficking analysis***

- Distinct imaging techniques will be used to study ENS vesicle trafficking **– 12 animals per group**:

e) FM1-43 imaging ***(in vitro)***: We will investigate how synaptic signalling and synaptic vesicle turnover (FM1-43) differ in ENS cultures derived from the different mouse models by using FM1-43 imaging and after distinct stimuli (chemical, electrical).

f) NTA analysis ***(in vitro*** and ***ex vivo)*** - To analyse pattern parameters of EVs: intensity fluctuations, surface geometry and shape of the particles as well as particle concentration to distinguish sub-populations of vesicles.

g) Manipulation of EVs by viral vector targeting in the ENS ***(in vivo)*** - AAVx.XFP carrying genetic modifiers to silence (shmiRNA) or overexpress molecules of interest, will be injected (i.v.) in our animal models to evaluate the role of EVs in the maintenance, organisation and function of the ENS and in CRC. No side effects are expected with AAV injections (mild discomfort). ***NB – Most of these experiments described above can be performed using the same group of animals as they are likely to cause transient and mild pain and discomfort.**

- ***Microbiota analysis***

Metagenomic DNA extraction from faecal, and tissue samples and molecular profiling/16S rRNA sequencing will be carried out to explore differences in the microbial community structure between experimental groups. We will examine the differences in microbial richness (e.g., *Chao1*) and diversity (e.g., *Shannon*). ***NB: The tissue collection will be achieved together with other experiments, in order to minimise unnecessary culling and animal use, so no extra animals will be used for this outcome measure.**

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

For the quantitative experiments, design has been based on ARRIVE guidelines and sample sizes have been set using power analysis https://www.statstodo.com/SSizCorr_Pgm.php. to determine the number of mice needed for each experiment, generally considering a difference between groups of at least 20%, power of 80% and significance level of 5%. For qualitative experiments, we will use the minimum number of mice to provide an accurate description based on previous publications and on our own experience, also from our previous PV.

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

1	Mouse	In-house breeding	(Young) adults	2118	Male and female	Wildtype, Mice with fluorescently labelled ENS cells, labelled extracellular vesicles from ENS cells, Transgenic mice with altered ENS, specifically modified ENS activity	

Provide justifications for these choices	
Species	Mice due to their anatomical, physiological, and genetic similarity to humans as well as their small size, ease of maintenance, short life cycle, and abundant genetic resources.
Origin	Sox10.CreERT2:R26tdTomato from a licensed non-commercial breeder in UK (but currently in house), Sox10.CreERT2:R26-GCaMP6f and *Wnt1.Cre:R26-hM4Di-DREADD* from licensed non-commercial breeders within Europe, NDRG4$^{fl/fl}$;Wnt1.Cre, NSE-Noggn and Hand2$^{fl/+}$;Wnt1.Cre are all in house, CD63 floxed from a licensed non-commercial breeder in USA
Life stages	Young adults (1-10 months) is most apt for optimal results
Number	Based on the literature, on our own and other collaborators experiences, we estimate that we will need a maximum of 12 mice per group for most outcome measures (high variability/spread expected because of the measurement method (for example, gut transit times vary a lot depending also on whether mice are very active or passive at the time of the experiment; tumor development/progression is very variable and tumors are divided in multiple grading groups, so you need multiple tumors per mouse for analysis (5.1 lid2e, 5. et al 2017; 5.1 lid2e, 5. et al 2021)). For FACS experiments, we will need max 9 mice per group (less tumor material needed, so less drop-out expected and spread is usually low (unpublished data)) and 6 mice for RNA experiments (less tumor material needed, so low drop-out expected and also low spread expected; RNA can partly also be obtained together with other outcome parameters (unpublished data; 5.1 lid2e, 5. et al 2017; 5.1 lid2e, 5. et al 2021)). In total, this will be maximally 2118 animals. For a more detailed overview of how this number is built up, we refer to the following Word document: Total number of mice appendix 2.
Gender	Both male and female mice will be used to minimise any gender-related variations in the experiment.
Genetic alterations	Mice with fluorescently labelled ENS (e.g. Sox10.CreERT2:R26tdTomato, Wnt1.Cre:R26tdTomato, Sox10.CreERT2:R26-GCaMP6f) will be used to study the interaction between the ENS and CRC. Specific ENS cells will be labelled and can thereby be easily followed in these models. Furthermore, activity and specific ENS cells can be studied. This will give us more insight into the effect of CRC on specific ENS cells and their activity and therefore we will get a better idea about possible interactions between these cell types. Mice with fluorescently labelled extracellular vesicles from ENS cells (e.g. CD63$^{X/X}$ crossed with ENS specific cre reporter lines e.g., Sox10.CreER^{T2} or Wnt1.Cre) can be used to study the involvement of extracellular vesicles in the interaction between the ENS and CRC. Transgenic mice with altered ENS (e.g., NDRG4fl/fl;Wnt1.Cre - Enteric neuronal-specific knockdown of NDRG4, Hand2f/+;Wnt1.Cre mice - more enteric neurons, and NSE-noggin - less enteric neurons) will be used to study whether neuron density or knockdown of NDRG4 (biomarker for CRC and specifically expressed in enteric neurons) affect CRC development/progression and which mechanisms play a role in this effect. Lines designed to specifically modulate ENS activity (e.g. Sox10.CreER^{T2}/Wnt1.Cre:R26-hM4Di-DREADD) will be used to study the effect of activation or inhibition of a specific subtype of ENS cells to the development/progression of CRC. This will give us insight into the role of ENS cells in CRC.

	Altogether, this may lead to possible pathways and/or targets that could be used for treatment in the future.
Strain	(mixed) C57BL/6J (e.g. NDRG4fl/fl;Wnt1.Cre, Hand2fl/+:Wnt1.Cre, Sox10.CreERT2, R26-hM4Di-DREADD, R26-GCaMP6f), (mixed) 129S4SvJaeJ (e.g. Hand2fl/+:Wnt1.Cre), (mixed) FvB (e.g. NSE-Noggin). The strain of the wildtype mice will be matched to the corresponding transgenic mice used in the procedure.

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

If mice have to undergo CT imaging, they need to be food deprived before performing the CT scan (max 16h and mostly in the light phase), so that no fecal pellets are present in the colon during imaging. Food will be given immediately when animals have undergone their CT scan.

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.

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

During the development of CRC or during CRC treatment animals might experience pain (see below). All possibilities to reduce pain, fear or suffering will be used. These might include use of appropriate analgesia (e.g. opiates (not in experiments of gut motility) or NSAIDs (not in experiments involving inflammation)) and anaesthesia (e.g. isoflurane) that do not affect gut motility, lead to gut abnormalities or affect the intestinal inflammatory response. However, use of analgesia is not expected based on experience with our past experiments, but pain will always be scored and evaluated to ensure proper management of the animals (if we cannot apply analgesia and animals experience discomfort, HEPs will be applied when necessary). Follow-up of the animals will be done regularly, at least daily, to ensure rapid notifications of signs of discomfort. Obviously, after experimental procedures e.g. injections/gavage, the animals will be followed up more frequently.

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

- *In vivo* experiments can cause distress or discomfort to the animals. Therefore, all animals will be frequently monitored for any signs of pain and distress. Changes to normal and provoked behaviour, movement, physical signs such as posture, respiration, skin and coat changes, inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist. Any mice exhibiting any deviation from the normal health and behaviour will be further monitored and treated accordingly.
- Genetically altered mice used in this appendix are not expected to show deleterious phenotypes.
- CRC mouse lines ($APC^{Min/+}$) and models (AOM and AOM/DSS) can develop side effects, such as pain, weight loss, diarrhoea/constipation and anal bleedings (no ulcers with perforation are expected).
- Radio- and Chemotherapies can induce necrosis, fibrosis and inflammation in irradiated tissues, and skin conditions may appear leading to loss of coat pigmentation depending on drug concentration. Furthermore, chemotherapy can induce side effects such as illness/sickness/nausea and reduction of appetite.

Explain why these effects may emerge.

- Most of the side effects mentioned above are unlikely in our non-diseased mice models (unless provoked), but can occur due to inflammation and/or progression of disease, development of polyps and alteration in intestinal microbiota.
- Radio- and chemotherapy can cause toxicity in normal/healthy tissues due to production of reactive oxygen/nitrogen species (ROS). This may result in immediate cell death, inflammation, tissue fibrosis and DNA damage in neighbouring tissues.

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

Animals will be frequently monitored by experienced staff and researchers. Our mouse colonies will be housed and maintained according to the basic guidelines for animal welfare. Working protocols will be adequately adjusted to benefit not only the research but also animal welfare. Experimental procedures/routes of administration, techniques that cause the least pain, suffering, distress and the shortest lasting harm will be preferred. Experimental procedures will be performed by using aseptic/sterile techniques, and drugs such as analgesics, anaesthetics and antibiotics will be administered whenever necessary. Examples of measures that we will use to prevent or minimise harm are minimizing radiotherapy and chemotherapy dosages and times to what is minimally required, extra hydration in case of diarrhea and boosting gels in case of high weight loss. Measures will be taken accordingly in the case any unexpected adverse effects might appear and animals will be immediately euthanized and experiments stopped in the case of unexpected severe signs of pain and discomfort.

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.

- All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist.
- Any animal will be immediately euthanised if it exhibits signs of suffering that is not transient or treatable, and compromises its normal behaviour.
- Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe discomfort and >20% for severe discomfort) that persist for 24 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision.
- Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled.
- Chemical CRC models: animals undergo euthanasia about 100 days after the beginning of the procedure. Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 2), “Wasting Syndrome” (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), or opportunist infections, the animals will be immediately euthanised.
- Radio- and Chemotherapies: adverse effects such as inflammation and skin conditions such as loss of coat pigmentation may appear. Animals can undergo moderate to severe adverse effects and will be culled if signs of severe discomfort persist such as facial expression score of 3, “Wasting Syndrome” (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), coat/skin condition 3 for at least 3 consecutive days, chronic diarrhea or opportunist infections.

Indicate the likely incidence.

For the CRC models, and in the wildtype x CRC mice that underwent chemo or radiotherapy (±10-15% incidence). The animals will be well monitored by experienced people, so that we are able to rapidly detect unforeseen adverse effects in early stages, whereby we aim to avoid reaching the humane endpoints.

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).

Appendix 2	Non-diseased gut (I)	APC$^{Min/+}$ mouse model (II)	AOM model (III)	AOM/DSS model (IV)
Discomfort model itself → Discomfort procedures per outcome measure ↓	**No discomfort**	**Mild**	**Mild**	**Moderate**
ENS structure /function /proliferation - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Gut physiology - Administration of substances: **mild** - Transit/motility assays: **mild** - Killing: **mild**	Mild	Moderate	Moderate	Moderate
Vesicle trafficking - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Microbiota analysis - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Intercellular communication - Administration of substances: **mild** - Killing: **mild**	Mild	Mild	Mild	Moderate
Development/progression of CRC - Administration of substances: **mild** - CT scans: **moderate** - Killing: **mild**	Moderate	Moderate	Moderate	Moderate

Appendix 2: Treatment	AOM model (III)	AOM/DSS model (IV)	Orthotopic model (V)
Discomfort model + treatment (chemo/radiotherapy)→ Discomfort procedures per outcome measure ↓	**Moderate**	**Moderate**	**Moderate**
Development/progression of CRC - Administration of substances: **mild** - CT scans: **moderate** - Killing: **mild**	Moderate	Moderate	Moderate

Figure 3: *Cumulative discomfort for disease models or treatment models (columns) combined with outcome measurements and corresponding procedures (rows).* The different mouse lines itself (described in Table 1) are not expected to have any harmful phenotype or display signs of discomfort.

Approximate percentages expected cumulative discomfort based on animal numbers per outcome measure and disease model:
Mild: 50%
Moderate: 50%
Severe: 0%

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	To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models to study CRC. In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied *in vivo*. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established *in vitro* culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis *in vivo.* We are also taking advantage of recently established collaborations (e.g. in the gastroenterology clinic (5.1 lid2h to obtain human intestinal specimens and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study cancer development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Reduction	Before embarking on procedures in animal research, we will collect evidence form *in vitro/in silico* procedures if possible to determine whether a candidate gene or molecule also provide insights on *in vivo* pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This can also be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals (mostly included in the previous PL (AVD5.1 lid2h), so not included in the current strategy). This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.
Refinement	Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no phenotype and/or useful results, experiments that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and/or well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached.

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.

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.

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.

N/A

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.

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.

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

Ex vivo (post-mortem) and *in vitro* experiments will be performed in tissue and cells collected from our experimental groups to further analyse the biology of the nervous system.

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.

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

☒ No > Describe the method of killing.

CO_2 inhalation and cervical dislocation

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

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

NON-TECHNICAL PROJECT SUMMARY

Country	NL	*
Language	NL	*
EU submission	yes [1]	*
Title of the project	Communicatie tussen het darmzenuwstelsel (i.e. enterisch	*
NTS identifier		
NTS national identifier	NTS202215867	
Duration of the project	60 **(in months)**	*
Keywords		*
Keyword 1	enteric nervous system	
Keyword 2	gastrointestinal tract	
Keyword 3	crosstalk	
Keyword 4	intestinal cancer cells	
Keyword 5	epigenetics	

Purpose(s) of the project *

Objectives and predicted benefits of the project

Objectives of the project *

Het algemene doel van dit project is tweeledig: (1) de organisatie en functie van het enterisch zenuwstelsel (EZS), ookwel het buikbrein genoemd, tijdens ziekte en gezondheid te doorgronden en (2) de interacties van dit buikbrein met andere organen en cellulaire systemen beter te begrijpen. Meer specifiek willen we met behulp van nieuwe modellen en reeds bestaande methodes de communicatie/interactie tussen het EZS en andere celtypes bestuderen, evenals de interacties binnen het EZS zelf. Daarnaast willen we ook proberen de mechanismen van het zenuwstelsel die de normale en verstoorde (bijv. darmontsteking, darmkanker) werking van het spijsverteringsstelsel reguleren bloot te leggen.

Potential benefits likely to derive from this project *

Het is een hele uitdaging om de organisatie en werking van het buikbrein te doorgronden omdat het gekenmerkt wordt door een ingewikkelde samenstelling en moeilijk te bereiken is, binnin de darmwand. Echter, dankzij samenwerkingen met andere laboratoria, heeft onze onderzoeksgroep momenteel de nodige kennis en methodologien om de cellulaire en moleculaire interacties in het EZS en tussen het EZS en andere cellen in detail te onderzoeken. Door de complexe samenstelling van het EZS en zijn veelvuldige interactie met andere celtypes zullen de bevindingen van onze studies ook als basis kunnen dienen voor onderzoek naar andere orgaansystemen en het centraal zenuwstelsel. Huidig onderzoek toont ook aan dat de darm, en daarin het EZS, als een toegangsdeur kan optreden in het ontstaan van verschillende ziektes die niet bekend staan als aandoeningen van het maag-darm

Predicted harms

In what procedures will the animals typically be used *

De muizen zullen enkele specifieke procedures ondergaan. Voor het eerste deel van ons onderzoek, het celkweek (in vitro) deel, zullen de dieren geen specifieke procedures ondergaan voordat we de darm isoleren om vervolgens onze celkweek systemen op te zetten. Daarnaast zullen we bepaalde ziektebeelden opwekken in de muizen. Dit zullen we op verschillende manieren aanpakken. We focussen daarbij op het nabootsen van dikke darmkanker en ontstekings-gerelateerde darmkanker. Deze ziektes kunnen we imiteren door bijv. een fout in het genetisch materiaal van de muizen te introduceren of door de muizen te injecteren met een kankerverwekkende stof, al dan niet in combinatie met een ontsteking verwekkende stof. Indien hieruit blijkt dat de muizen succesvol tumoren ontwikkelen, zullen we pas over kunnen gaan tot het toepassen en bestuderen van de

Expected impacts/adverse effects on the animals *

We verwachten dat het grootste deel van de muizen geen tot licht ongerief zullen ondervinden. Als gevolg van hun genetische afwijkingen in het EZS, kunnen de muizen mogelijk last ondervinden van een veranderde darmbeweging. Wanneer de dieren onderworpen worden aan chirurgische procedures, procedures om stoffen te injecteren en/of de verscheidene ziektebeelden na te bootsen, zullen zij een licht tot matig ongerief kunnen ondervinden. Dit voornamelijk als gevolg van een verstoorde darmbeweging, ontstekingen, bloedverlies en gewichtsverlies. Tot slot kan er ook matig ongerief optreden op het ogenblik dat de muizen met een veranderd EZS onderworpen worden aan bepaalde ziektemodellen en behandeld worden met radio/chemotherapie, alsook wanneer ze gebruikt worden om de ontwikkeling en groei van tumoren live te volgen d.m.v. CT-scans. *

Fate of animals kept alive

Reasons for the planned fate of the animals after the procedure *

Voor het beoordelen van verscheidene darmfuncties (bv. darmbeweging/ darmdoorlaatbaarheid) worden dezelfde dieren op verschillende tijdstippen onderworpen aan verschillende experimenten. Ook wanneer tumor aantal en grootte bepaald wordt, zullen dezelfde dieren op verschillende tijdstippen CT-scans ondergaan. Wanneer de dieren alle experimentele procedures volledig doorlopen hebben, zullen ze gedood worden en zal het materiaal van de overleden dieren gebruikt worden voor verder onderzoek om bijv. de structuur van het EZS, de ontwikkeling en progressie van kanker/ontsteking te bestuderen.

Application of the Three Rs

1. Replacement *

We hebben recentelijk nieuwe samenwerkingen opgezet, waardoor we a.d.h.v. humaan materiaal reeds de betrokkenheid van het EZS in de verschillende ziektebeelden kunnen onderzoeken. Daarnaast, om de mechanismen volledig tot in detail uit te diepen zullen we ook gebruik maken van verschillende celkweekmodellen. Dit laat ons toe om op grotere en preciezere schaal de details te onderzoeken. Ons lab heeft ondertussen veel ervaring met het opzetten van deze celkweekmodellen. Hierin bestuderen we kandidaatgenen/pathways, voordat we deze verder onderzoeken m.b.v onze muismodellen. Echter kunnen we niet volledig afstappen van diergebruik, aangezien deze systemen de volledige complexiteit van het lichamelijk functioneren omvatten. Desalniettemin zullen we de meest optimale modellen gebruiken. Aangezien we in de muismodellen de volledige

2. Reduction *

Voor we beginnen aan onze dierexperimenten, zullen we informatie verzamelen a.d.h.v. celkweek, (publieke) data en in silico (computer gebaseerde) analyses, zodat we gerichtere experimenten kunnen opzetten. We zullen de dierexperimenten hiermee zo beperkt mogelijk kunnen houden. Indien de muizen niet de juiste waarneembare in of uitwendige kenmerken (zoals bijv. tumorvorming) vertonen, of de eerste experimenten niet de gehoopte resultaten opleveren, zullen we niet verder gaan met deze modellen (Go/No-go). Omdat we over verschillende onderzoeksprojecten gelijkaardige modellen en procedures gebruiken, kunnen we data gebruiken voor verscheidene projecten, waardoor we ook minder dieren nodig hebben. We zullen proberen om onze fok zo efficient mogelijk op te zetten om zo te zorgen dat we overbodige kweken en een onnodig overschot van nakomelingen

3. Refinement *

De gekozen modellen en procedures zijn essentieel om nieuwe inzichten te krijgen in humane ziektes die het EZS aantasten. Door modellen te gebruiken waarbij veranderingen in het EZS op een tijdelijke en/of heel specifieke manier geïntroduceerd worden, zullen de bijwerkingen beperkt blijven. Alle protocollen, modellen en behandelingen die onder deze licentie worden gebruikt, zijn standaard en werden eerder en uitgebreid uitgevoerd door leden van ons laboratorium, medewerkers en/of goed gedocumenteerd in relevante literatuur. Pilotstudies zullen vooraf worden uitgevoerd om te bepalen wat de minimale dosis en blootstellingstijd is die een effectieve werking vertoont met minimale neveneffecten. Ook zullen we ervoor zorgen dat de dieren zo weinig mogelijk bijwerkingen/ stress ondervinden van de procedures. Een mogelijkheid hiertoe is de toediening van verdoving (keuze

Explain the choice of species and the related life stages *

In dit onderzoeksproject is gekozen voor muizen, niet alleen omwille van hun gebruiksgemak (e.g. klein, groepshuisvesting, grote beschikbaarheid aan modellen), maar met name vanwege hun enorme anatomische, fysiologische, en genetische gelijkenis met mensen. Bovendien zijn er veelvuldig toegepaste en valide onderzoeksprotocollen voor het werken met cellen en weefsels van deze dieren en voor het werken met levende dieren. Er zal voornamelijk met volwassen dieren gewerkt worden, vanwege de beschikbaarheid van valide protocollen voor deze leeftijdscategorie voor onze onderzoeksdoelen, en vanwege de betere vertaalbaarheid naar de menselijke situatie. Driedimensionale celkweek modellen van het EZS zullen verkregen worden uit muizenembryo's of pasgeborenen, zoals eerder toegepast. De driedimensionale celkweek modellen kunnen langdurig

Project selected for Retrospective Assessment

Project selected for RA? *	no [0]
Deadline for RA	

Reasons for retrospective assessment

Contains severe procedures	
Uses non-human primates	
Other reason	

Explanation of the other reason for retrospective assessment

Additional fields

National field 1

National field 2	
National field 3	
National field 4	
National field 5	
Project start date	
Project end date	
Project approval date	
ICD code 1	
ICD code 2	
ICD code 3	
Link to the previous NTS version outside the EC system	

Advies aan CCD

Datum 04 juli 2022
Betreft Advies Secretariaat over Aanvraag projectvergunning Dierproeven AVD202215867

Instelling:	5.1 lid2h
Onderzoeker:	5.1 lid2e
Project:	The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease
Aanvraagnummer:	AVD202215867
Betreft:	Nieuwe aanvraag
Categorieën:	Fundamenteel onderzoek

1 Inzicht in aanvraag en de eventuele knelpunten en risico's

Proces	Er zijn geen vragen gesteld aan de DEC. Aan de aanvrager is gevraagd over de NTS: Uw aanvraag draait om het enterisch zenuwstelsel. Kunt u in de NTS helder uitleggen wat dit is? Daarnaast bevat de NTS veel lastige termen die niet begrijpelijk zullen zijn voor een leek. Kunt u de NTS beter navolgbaar maken? U benoemt bij vervanging het 5.1 lid2h De NTS dient echter anoniem te zijn. Kunt u het 5.1 lid2h uit de NTS halen? Kunt u mild ongerief veranderen naar licht ongerief? Over bijlage 2 van de dierproeven: Kunt u in bijlage 2 van de dierproeven bij C aanvullen hoe lang de dieren geen voedsel krijgen voordat ze een CT scan krijgen? Wanneer in bijlage 2 van de dierproeven uit de pilot waarin de dosis chemo-en radiotherapie wordt onderzocht blijkt dat ernstig ongerief wordt voorkomen dan zal dit een 'go' moment zijn. Hierdoor zullen de dieren in het aangevraagde onderzoek geen ernstig ongerief ondervinden. Kunt u bij bijlage 2 van de dierproeven bij F de classificatie van het cumulatieve ongerief in procenten aanpassen?

Naam proef	Diersoort	Stam	Aantal dieren	Herkomst
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres				
	Muizen (Mus musculus)		1.322	Dieren die voor onderzoek gefokt zijn
3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer				
	Muizen (Mus musculus)		2.118	Dieren die voor onderzoek gefokt zijn
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases				
	Muizen (Mus musculus)		2.385	Dieren die voor onderzoek gefokt zijn

Huisvesting en verzorging anders dan Bijlage III Richtlijn

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

Citaat: If mice have to undergo CT imaging, they need to be food deprived before performing the CT scan, so that no fecal pellets are present in the colon during imaging. Food will be given immediately when animals have undergone their CT scan.

Gebruik van mannelijke en vrouwelijke dieren

3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases

Muizen (Mus musculus) Er worden zowel mannelijke als vrouwelijke dieren gebruikt.

Locatie uitvoering experimenten	- Alle proeven vinden plaats in een instelling van een vergunninghouder. - Er zijn geen problemen bekend met de vergunninghouder.

2 DEC advies

DEC-advies	Citaat vraag DEC aan de aanvrager (vraag 38): Waarom worden dieren aangevraagd voor experimenten die ook omschreven zijn binnen vorig PV, welke nog loopt? Worden de experimenten beschreven in vorig PV niet meer uitgevoerd, hoewel er nog een jaar looptijd is?

	Citaat antwoord aanvrager (vraag 38): We hebben hier enkel dezelfde diermodellen aangevraagd als in ons vorige PV als er nieuwe outcome parameters zijn toegevoegd die niet in onze huidige PV staan of we hebben voor dezelfde uitcome parameters nieuwe diermodellen aangevraagd. Een uitzondering hierop zijn de dieren voor therapie. Deze experimenten zijn niet meer haalbaar in de lopende PV en zijn dus verplaatst naar deze aanvraag. Citaat vraag DEC aan de aanvrager (vraag 47): 10 CTs leidt tot een cumulatieve bestralingsdosis (whole body) van minimaal 3 Gy. Welk effect heeft dit op de uitleesparameters van de desbetreffende experimenten? En op het ongerief? Wat is de stralingsgevoeligheid van de muizenstammen die u wilt gebruiken? Citaat antwoord aanvrager (vraag 47): Ongerief zal zoals beschreven, moderate-to-severe zijn. De pilootexperimenten die onder de huidige PV beschreven staan, zullen echter gebruikt worden om het effect van de therapie te bestuderen en het ongerief zoveel mogelijk te beperken. Om de stralingsgevoeligheid, de dosis en frequentie te bepalen zullen we samenwerken met andere onderzoekers die vergelijkbare experimenten eerder hebben uitgevoerd. Citaat vraag DEC aan de aanvrager (vraag 53): U geeft aan dat bestraling en behandeling met chemotherapie van de muizen tot “moderate (to severe)’ ongerief kan leiden. Verwacht u hier maximaal ernstig of maximaal matig ongerief? Citaat antwoord aanvrager (vraag 53): We hebben hier op dit moment nog maximaal ernstig ongerief beschreven. We gaan echter met de pilootstudies onder onze huidige PV deze procedures optimaliseren en daarmee verwachten we, maar zijn we niet geheel zeker dat, het ongerief terug te kunnen brengen naar matig ongerief. Citaat vraag DEC aan de aanvrager (vraag 4, ronde 2): De 5.1 lid2h kan geen ethische inschatting maken over het therapie gedeelte (ongerief, humane eindpunten) aangezien wordt aangegeven dat dit afhankelijk is van de uitkomst van pilootstudies die nog dienen te gebeuren op het huidig lopende PV (en daarom nu als maximaal ernstig wordt ingeschat). Echter op vraag 38 geeft u als antwoord dat alle therapie studies van huidig lopend PV niet meer zullen uitgevoerd worden en daarom zijn meegenomen in dit PV. Dit lijkt dus een belangrijke tegenstrijdigheid te zijn. Bovendien is het feit dat dieren ernstig ongerief kunnen ondervinden een belangrijk ethisch dilemma in deze aanvraag. Daardoor kan de

	5.1 lid2h over dit gedeelte nu geen beslissing nemen. We stellen daarom voor om OF deze pilootstudies mee te nemen in huidige aanvraag met een duidelijke go/no-go beslissing naar de eigenlijke therapie studies (inclusief zo veel mogelijke beperking van het ongerief) OF de therapie-studies uit de aanvraag te halen. Citaat antwoord aanvrager (vraag 4, ronde 2): Zoals correct opgemerkt zullen enkel de pilootstudies, maar niet de officiële therapiestudies uitgevoerd worden op het lopende PV. Deze huidige PV loopt namelijk nog 1,5 jaar, waarin wij verwachten deze pilootstudies uit te voeren. Daarom zijn deze pilootstudies dus niet meer opgenomen in deze nieuwe PV aanvraag. De therapiestudies zelf krijgen we niet meer uitgevoerd in de komende 1,5 jaar, waardoor deze wel opgenomen zijn in deze nieuwe PV. Om ervoor te zorgen dat er een goede ethische overweging gemaakt kan worden hebben we nu een duidelijker go/no-go moment toegevoegd (appendix 2, p.5). De pilootstudies uit de lopende PV zullen als go/no-go moment gelden voor de therapiestudies in deze PV. In het geval het niet mogelijk blijkt (op basis van de pilootstudies) om de therapiestudies met matig ongerief uit te voeren, zullen deze experimenten vervallen (max 432 dieren). Het ongerief zal hierdoor zoveel mogelijk beperkt worden en wordt geschat op moderate. Citaat C10: Een deel van de dieren zal maximaal 16 uur zonder voedsel gezet worden, zodat er geen voedsel in de darmen zit bij het uitvoeren van de CT-scans. De 5.1 lid2h is er verder van verzekerd dat voldaan wordt aan huisvesting en verzorging volgens de richtlijn op basis van de daartoe strekkende verklaring (in duplo) van zowel de vertegenwoordiger van de vergunninghouder, als de aanvrager onder respectievelijk punt 6 der ondertekening van de aanvraag en punt F in de bijlagen. Citaat C11: De 5.1 lid2h acht het ongerief grotendeels realistisch ingeschat. In het voorstel wordt aangegeven dat in totaal 68% van de dieren licht, 23% matig en 9% ernstig ongerief zal kunnen ervaren. De 5.1 lid2h vindt dat ernstig ongerief binnen deze aanvraag niet gerechtvaardigd is. Uit de antwoorden van de onderzoekers (vraag 4, tweede ronde) is namelijk gebleken dat er op een lopende vergunning pilotstudies uitgevoerd zullen worden waarbij de optimale omstandigheden voor de experimenten met potentieel ernstig ongerief bepaald worden. Wanneer de uitkomst van de pilotstudies is dat er ernstig ongerief zal optreden, is dit aangemerkt als een 'no-go' voor de betreffende vervolg-experimenten binnen deze aanvraag. Ethische afweging van de DEC: Citaat:

	1. Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan? 2. -Waarden die voor de proefdieren in het geding zijn: substantieel nadeel -Waarden die voor onderzoekers bevorderd worden: reëel voordeel. -Waarden die voor de medische wetenschap bevorderd worden: reëel voordeel. De 5.1 lid2h is van mening dat de belangen van onderzoekers/wetenschappelijke instituten, binnen het project 'The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease' zwaarder wegen dan de belangen/waarden van de proefdieren/dierbeschermingsorganisaties, mits ernstig ongerief wordt voorkomen. Voor de betrokken proefdieren leiden de beschreven proeven tot de dood na, in het voorstel aangegeven licht ongerief voor 68% van de dieren, matig ongerief voor 23% van de dieren en ernstig ongerief voor 9% van de dieren. De 5.1 lid2h is echter van mening dat ernstig ongerief voorkomen dient te worden, omdat er een go-no go is ingebouwd door de onderzoekers tussen deze aanvraag en een reeds lopende vergunning. Wanneer uit de pilotstudies onder de lopende vergunning blijkt dat ernstig ongerief niet vermeden kan worden, zullen de betreffende experimenten in de huidige aanvraag geen doorgang vinden (zie antwoord op vraag 4 uit de tweede ronde vragen). De dieren worden door de experimenten in hun welzijn geschaad. De integriteit van de dieren zal worden aangetast door de experimentele handelingen (injecties (o.a. voor het induceren van genetische veranderingen en het toedienen van chemische middelen), inductie van darmkanker (via injecties, of via colonoscopie), CT-scans (met bijbehorende voedseldeprivatie), bestraling, chemotherapie, verschillende manieren voor het induceren van darminflammatie (worminfectie, dextraan natriumsulfaat (DSS)), laparotomie, het veranderen van de darmflora (antibioticumkuur gevolgd door fecaal transplantaat, vasten) en het leven met de gevolgen daarvan gedurende de proeven en de opoffering aan het eind daarvan. Indien de doelstellingen bereikt worden, zal dit project echter leiden tot meer inzicht in de communicatie tussen het enterische zenuwstelsel en andere celtypen in homeostase of onder pathologische omstandigheden

zoals inflammatie, kanker of veranderingen in de microbiota. Daardoor zullen aandoeningen aan de darm mogelijk beter begrepen worden en worden deuren geopend naar nieuwe interventies.

Het is aannemelijk dat de doelstelling behaald zal worden. De onderzoekers zullen zoveel mogelijk trachten het lijden van de dieren te beperken.

3. De 5.1 lid2h beantwoordt de centrale morele vraag 'Weegt in het voorgestelde onderzoek naar de organisatie en functie van het enterisch zenuwstelsel tijdens ziekte en gezondheid en de interacties van het enterisch zenuwstelsel met andere organen en cellulaire systemen, op tegen de opoffering, het ongerief, en de aantasting van de integriteit dat de dieren (muizen) wordt aangedaan?' bevestigend.

De 5.1 lid2h onderschrijft de integriteit en intrinsieke waarde van het dier en heeft oog voor het te ondergane ongerief van de proefdieren. Naar haar mening weegt het reële belang van dit project, en meer specifiek de belangen van de onderzoekers en het betreffende onderzoeksgebied zwaarder dan de voorgestelde schending van integriteit, het te berokkenen ongerief en opoffering, mits ernstig ongerief wordt vermeden.

De 5.1 lid2h is van mening dat de voorgestelde experimentele opzet en de uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en dat de gekozen strategie en de voorgestelde experimentele aanpak kunnen leiden tot het behalen van de doelstelling binnen het kader van het programma. De onderzoekers beschikken over de benodigde kennis en technische expertise, zoals duidelijk uit hun voorstel blijkt. Er is geen sprake van duplicatie.

In de gekozen strategie wordt op bevredigende wijze tegemoetgekomen aan de vereisten van vervanging, vermindering en verfijning. De 5.1 lid2h is ervan overtuigd dat de aanvrager voldoende maatregelen treft om zowel het ongerief van de dieren als het aantal benodigde dieren tot een minimum te beperken. Er zijn voldoende go/no-go momenten voorzien om onnodige dierproeven te vermijden. De 5.1 lid2h is ervan overtuigd dat er geen alternatieven zijn, waardoor deze dierproef met minder ongerief of met minder, dan wel zonder levende dieren zou kunnen worden uitgevoerd. Wel is zij van mening dat ernstig ongerief vermeden kan en moet worden.

Op grond van deze overwegingen beschouwt de 5.1 lid2h de voorgestelde dierproeven in het projectvoorstel "The crosstalk between the enteric

	nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" als ethisch gerechtvaardigd, mits ernstig ongerief wordt vermeden. Derhalve voorziet de 5.1 lid2h het projectvoorstel "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" van een positief advies onder voorwaarde dat ernstig ongerief voor de dieren vermeden zal worden. De DEC heeft extern advies ingewonnen bij - de aanvrager is om aanvullingen gevraagd De DEC heeft een groot aantal vragen gesteld, opgenomen in 2 bijlagen van het DEC advies. Het DEC advies is Verlenen onder voorwaarden De onderzoekers geven aan dat uitkomsten van pilotstudies op een lopende vergunning zorgen voor een go-no go voor het deel van de experimenten die hier worden aangevraagd. Daarbij is een no-go van toepassing wanneer uit de pilot-experimenten blijkt dat ernstig ongerief niet vermeden kan worden. Het uitgebrachte advies is niet gebaseerd op consensus. Citaat: Het uitgebrachte 5.1 lid2h advies is tot stand gekomen op basis van een meerderheidsstandpunt. Een DEC-lid nam een minderheidsstandpunt in. De overwegingen hiervoor waren: (1) het grote aantal muizen staat nauwelijks in verhouding tot de praktische baten van het onderzoek; aangezien het fundamenteel onderzoek betreft is het onzeker of hier nieuwe therapieën uit voort gaan komen; (2) het gebruik van genetisch gemodificeerde muizen. De volgende dilemma's zijn gesignaleerd door de DEC: Citaat: Het uitgebrachte 5.1 lid2h advies is tot stand gekomen op basis van een meerderheidsstandpunt. Een DEC-lid nam een minderheidsstandpunt in. De overwegingen hiervoor waren: (1) het grote aantal muizen staat nauwelijks in verhouding tot de praktische baten van het onderzoek; aangezien het fundamenteel onderzoek betreft is het onzeker of hier nieuwe therapieën uit voort gaan komen; (2) het gebruik van genetisch gemodificeerde muizen.

3 Kwaliteit DEC advies

Kwaliteit DEC-advies	
Het DEC advies is helder en navolgbaar. Bij de beantwoording van de beoordelingsvragen verstrekt u een heldere onderbouwing. De CCD had bij vraag C10 graag de mening van de DEC gezien. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. Het valt op dat u zeer veel vragen hebt gesteld. Hoewel dit de kwaliteit van de aanvraag verbetert, wekt het ook de suggestie van meeschrijven, wat onwenselijk is. U heeft in uw advies een voorwaarde voorgesteld. Het is de CCD duidelijk waarom u de voorgestelde voorwaarde wilt stellen. Ze neemt deze voorwaarde niet over omdat de aanvrager zelf in haar aanvraag in bijlage 2 van de dierproeven aangeeft de pilot als go- no go moment te hanteren. Er is over dit go-no go moment wel een opmerking in de beschikking opgenomen. De CCD stelt het op prijs dat goed navolgbaar in uw advies is opgenomen dat het advies is gebaseerd op een meerderheidsstandpunt, en waarop het minderheidsstandpunt op gebaseerd is. De CCD had wel graag de onderbouwing gezien wat voor het minderheidsstandpunt doorslaggevend was wat betreft het gebruik van genetisch gemodificeerde muizen. Ook stelt de CCD het op prijs dat bij E3 goed navolgbaar is weergegeven waar discussie over is geweest tijdens de bespreking van deze aanvraag.	

4 Inhoudelijke beoordeling

Doelstelling Doelstelling	Citaat: The ultimate goal of this project is to get a better understanding of the molecular pathways that orchestrate the organisation and function of the ENS, and its integration with other tissues (e.g., brain) and systems (e.g., immune, vascular and epithelial systems) during physiological challenges (e.g. microbiota) and pathological challenges (e.g. gastrointestinal diseases like cancer, worm infection). This will bring us closer to understand how the ENS participates in the onset and progression of diseases that affect the gastrointestinal tract, and therefore will eventually contribute to the development of new targets and therapies to tackle gastrointestinal disorders. For this purpose, we aim to develop a holistic and advanced approach to study the influence of the intestinal environment on ENS transcriptome, morphology and physiology, both in health and disease, for which we will use well-established murine models and procedures.
Wetenschappelijk en maatschappelijk belang	Citaat: Relevance for research: Research interest focusing on the gut has increased in recent years because of the clinical and biological relevance of this organ in several diseases. More specifically, it has been recently shown (by our and other groups) that the ENS interacts with multiple

	systems and has been implicated in the onset and progression of many diseases that not only affect the gastrointestinal tract but also the CNS (5). Due to the intricacy of its network and close proximity with many other tissues, studying the nervous system of the gut is challenging and requires expertise from distinct fields. Our group and collaborators have all necessary tools and skills to thoroughly dissect the cellular and molecular pathways that underlie the ENS crosstalk with other systems in various conditions. Given the similarities in composition, organisation and function between the ENS and the CNS, investigating the role of the ENS in homeostasis and disease would allow scientists to take the complexity of the gut to study other organs. We expect to unite different fields to unravel many other biological questions raised in this project and contribute for the consolidation of enteric neuroscience. Relevance to the patient: Increasing evidence shows that the ENS might be the "entrance door" for several pathologies, including those affecting brain homeostasis. In neurodegenerative diseases, for instance, ENS phenotype and gastrointestinal malfunction have been shown to precede brain and/or motor symptoms by several years (5). Moreover, the ENS is likely to contribute to carcinogenesis, as both neo-neurogenesis and perineural invasion are unfavourable factors for CRC patients, which indicates that their survival rate is negatively affected by the higher nerve density in the tumour area (i.e. neo-neurogenesis) and invasion of tumour cells throughout nerve fibres (i.e. perineural invasion) (6). With confidence, it is conceivable to suggest that the participation of the ENS in diseases that affect the gastrointestinal tract and systems beyond it (i.e., gut-brain axis) deserves more attention. We aim to study whether the ENS functions as a key player in the maintenance of the fitness of the intestinal microenvironment, and its crosstalk with the brain. Furthermore, we consider the ENS as a potential target for therapies in diseases that affect the homeostasis of the gastrointestinal tract in humans. Our prospect is thus to unravel the role of ENS behaviour in homeostasis and under circumstances that disturb the equilibrium of the healthy gastrointestinal tract. Therefore, we will include relevant animal and in vitro models to mimic human conditions, (e.g., cancer and helminthic infections), that impair intestinal function. By using advanced technology to study the ENS and powerful insights from experts in gastroenterology, we intend to identify novel cellular and molecular mechanisms, and biomarkers (e.g., EV-derived small molecules, non-coding miRNAs) that translate the onset of diseases progression and/or (response to) treatment.
Onderbouwing wetenschappelijk en maatschappelijk belang	Het belang is voldoende uitgewerkt en onderbouwd.

Wetenschappelijke kwaliteit Kwaliteit aanvrager/ onderzoeksgroep en onderzoek	Citaat C7 uit het DEC advies: Voor zover de 5.1 lid2h kan beoordelen zijn de kennis en kunde van de onderzoeksgroep adequaat gezien de jarenlange ervaring met proefdiermodellen voor het bestuderen van het enterisch zenuwstelsel, wetenschappelijke output, de verworven interne- en externe financiering alsmede de aandacht voor de drie V's onder meer geïllustreerd aan de hand van publicaties in tijdschriften als Nature Reviews Gastroenterology & Hepatology, American Journal of Physiology Gastrointestinal and Liver Physiology, J Neurogastroenterology and Motility en Glia. Het Secretariaat heeft geen reden hieraan te twijfelen.

3V's

Vervanging	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: If possible, we will perform the experiments using established in vitro cell lines. Only when these techniques are not optimal to investigate our research questions, or if they do not work, will we use the primary cell cultures to investigate our hypothesis. For example, no ENS cell lines are available to study our research questions, therefore primary cell cultures need to be used. We aim to validate our data using cell cultures derived from human intestinal tissue specimens. However, compared to the high murine cell yield and tissue availability, a limited number of human tissue samples is available and the cell yield is also narrow, thereby limiting the possibility to adequately and preferentially use human samples. Furthermore, human samples cannot be easily manipulated to have the same potential as our mouse lines.

	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models to study CRC. In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied in vivo. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established in vitro culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis in vivo. We are also taking advantage of recently established collaborations (e.g. in the gastroenterology clinic (5.1 lid2h to obtain human intestinal specimens and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study cancer development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.

	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Citaat: To address genetic and physiological pathways that are crucial to human clinical studies, it is necessary to utilize animal models, for instance the ones described in this document (such as H. poly infection models). In addition, studies on the microbiota, brain and ENS behaviour can only be possible when studied in vivo. However, our current and future experiments take extensive advantage of alternative methods to animals, such as well-established in vitro culture systems of mammalian cells that are unquestionably a significant source of replacement. Our lab has also acquired vast experience in using adeno-associated viruses to target ENS cells in the adult mice. This is a significant alternative to the excessive breeding/surgery used to alter genetic material into the ENS. The use of animals will also be partly replaced by first assessing candidate molecules/genes on ENS and organoid cultures prior to any further analysis in vivo. We are also taking advantage of recently established collaborations in the gastroenterology clinic (5.1 lid2h to obtain human intestinal specimens (IBDs and IBS) and investigate the participation of the ENS in human gastrointestinal diseases to replace part of the animal usage. However, due to difficulties obtaining enough and suitable material and the impossibility to study disease development and progression and cross-talk between systems/cell types, animal experiments are still essential to answer our research questions.
Verminderen	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: We will limit the number of animals by isolating different cell types from the intestinal tract of the same mouse and by using animals that come from breedings carried out for the procedures in appendix 2/3, but cannot be used there.

	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: Before embarking on procedures in animal research, we will collect evidence form in vitro/in silico procedures if possible to determine whether a candidate gene or molecule also provide insights on in vivo pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This can also be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals (mostly included in the previous PL (AVD5.1 lid2h), so not included in the current strategy). This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.

	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Citaat: Before embarking on any procedures in animal research, we are collecting as many evidence as it is necessary to determine whether a candidate gene or molecule also provide insights on in vivo pathways. For this, we have been using relevant literature and database collected by us and other groups to select possible candidates to be used in our research. Additionally, our lab also shares the same approach (models) among the research projects, allowing more data to be collected from one animal/model and by consequence, reducing animal usage. This is also can be more effective and improved by taking advantage of insights and consultations with animal staff, carefully controlling breeding, numbers, health and phenotype of our colonies. Our lines, where possible, will be maintained as homozygous, reducing the excessive need of large offspring. In other cases, breeding strategies will be carefully designed to generate offspring containing heterozygous or wildtype littermates that match age and sex. Pilot studies (if required when information needed is not available) will be pre-screened in small studies to reduce the need of excessive use of animals. This appendix is also valuable to reduce the need for extensive animal usage as we are optimally harvesting several tissues, fluids and cell types per individual mice. Integration in animal research is essential to strength the information collected from minimum resources.

Verfijnen	
	3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres: Citaat: We do not expect to culture cells from any animals that display harmful phenotypes. Our experimental in vitro approach will allow us to gather very specific insights prior to doing any in vivo procedures.
	3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer: Citaat: Mice and procedures chosen for this license are fundamental to provide insights into human pathologies that affect the ENS. When appropriate, we will minimise side effects associated with models by using inducible or conditional alleles from a specific cell type rather than the whole mouse. Experiments that cause less discomfort will be prioritised and in case of no phenotype and/or useful results, experiments that might cause greater discomfort will not be further performed. To reduce stress during manipulation, we will follow national practice guidelines to minimise animal discomfort. All protocols, models, treatments used under this license are standard and were previously and extensively performed by members of our lab, collaborators and/or well-stablished in relevant literature. Pilot studies will also be pre-screened to obtain minimum dose and exposure time that will be effective with minimal potential suffering. All surgical work will be performed in accordance with the standard principles established by the Dutch authorities. When necessary, analgesia and anaesthesia will be provided using protocols supervised by animal staff. Mice will be sacrificed if one of the humane endpoints defined in the working protocol is reached.
	3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases: Zie bijlage 3.4.3.2. Citaat: Discomfort level in inflammatory, H. poly models might be mild to moderate (DSS). In the case of severe discomfort, mice will be immediately sacrificed.

Hergebruik	Er is geen sprake van hergebruik van dieren.

Naam proef	Worden de dieren gedood?	Doden volgens richtlijn?
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres	Ja	volgens de richtlijn.
3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer	Ja	volgens de richtlijn.
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases	Ja	volgens de richtlijn.

Naam proef		
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres	HEP: Worden niet verwacht	
Muizen (Mus musculus)	Ongerief: 100,0% Licht	

3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer	HEP: 10-15%	Citaat: • All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persist. • Any animal will be immediately euthanised if it exhibits signs of suffering that is not transient or treatable, and compromises its normal behaviour. • Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe discomfort and >20% for severe discomfort) that persist for 24 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision. • Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled. • Chemical CRC models: animals undergo euthanasia about 100 days after the beginning of the procedure. Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 2), "Wasting Syndrome" (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), or opportunist infections, the animals will be immediately euthanised. • Radio- and Chemotherapies: adverse effects such as inflammation and skin conditions such as loss of coat pigmentation may appear. Animals can undergo moderate to severe adverse effects and will be culled if signs of severe discomfort persist such as facial expression score of 3, "Wasting Syndrome" (combination of body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), coat/skin condition 3 for at least 3 consecutive days, chronic diarrhea or opportunist infections.

Muizen (Mus musculus)	Ongerief: 50,0% Matig 50,0% Licht	Omdat er een go-no go moment als voorwaarde in de beschikking wordt opgenomen zal ernstig ongerief voorkomen worden.
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases	HEP: 10-15%	Citaat: All animals will be frequently monitored for any signs of pain and distress. Changes to normal intrinsic and provoked behaviour, movement, physical signs such as posture (hunching/huddling), respiration, skin and coat changes (piloerection, excess or absence of grooming), inactivity, inappetence, alterations in body weight, food and water uptake, inflammation of injection sites will result in euthanasia if one of these signs persists for longer than 24 hours. • Any animal will be immediately euthanised if it exhibit signs of suffering that is not transient or treatable and compromises its normal behaviour. • Any animals that display sudden body weight loss (>15% for moderate discomfort or cumulative severe disomfort and >20% for severe discomfort) that persist for 48 hours should be killed. In the case of weight loss of greater that 15%, veterinarian will be immediately contacted for health evaluation and fate decision. • Genetically altered mice used in this appendix are not expected to show any deleterious phenotype, but in case they do, they will be immediately killed. • Any animal that does not fully recover from the surgical procedure within the first 24 hours will be culled. • Inflammation models: Intestinal prolapses and presence of blood in the stool may occur. In the case of chronic stool blood loss (at least 3 consecutive days), anemia, prolapse progression (for 3 consecutive days), obstipation, facial expression (score 3), “Wasting Syndrome” (body weight loss, body condition score 1, abnormal grooming, inappetance, inactivity), chronic diarrhea or opportunist infections, the animals will be immediately euthanised. • In the case severe discomfort occur in multiple mice during the experimental procedures described abobe, the experiments will be ended and the animals euthanised. • H. poly - Some weight loss is expected but mice carrying other mutations may show more severe

		responses. Any mice showing weight loss of 15% of starting weight or diarrhoea, bloody stools, rectal prolapse, abdominal discomfort and bloating for more than 24h will be killed.
Muizen (Mus musculus)	Ongerief: 35,0% Matig 65,0% Licht	

5 Samenvatting

5.2 lid1

In bijlage 2 van de dierproeven zullen dieren voordat ze een CT scan krijgen 16 uur worden gevast. 5.2 lid1

Het uitgebrachte advies van de DEC is tot stand gekomen op basis van een meerderheidsstandpunt. 1 lid nam het minderheidsstandpunt in. De overwegingen hiervoor waren het aantal dieren tov het fundamentele onderzoek en de onzekere uitkomst of hier nieuwe therapieën uit voortkomen en het gebruik van genetisch gemodificeerde muizen.

Daarnaast is er in de DEC gediscussieerd over de haalbaarheid in de gestelde tijdsduur en over het ernstige ongerief dat dieren kunnen ondergaan. De DEC stelt voor om de volgende voorwaarde op te nemen in de beschikking:

Dat de dieren niet meer dan matig ongerief zullen ondervinden. De onderzoekers geven aan dat uitkomsten van pilotstudies op een lopende vergunning zorgen voor een go/no go voor het deel van de experimenten die hier worden aangevraagd. Daarbij is een no/go van toepassing wanneer uit de pilot-experimenten blijkt dat ernstig ongerief niet vermeden kan worden.

5.2 lid1

Daarbij geeft de aanvrager in bijlage 2 van de dierproeven van het aangevraagde onderzoek zelf ook aan deze pilot als go/no go te hanteren. Ze schatten zelf het ongerief nog wel in op ernstig. Het Secretariaat heeft gevraagd dit in de bijlage dierproeven aan te passen. Met het opnemen van de voorwaarde in de beschikking wordt ernstig ongerief bij de dieren voorkomen.

6 Voorstel besluit incl. voorstel geldigheidsduur van de vergunning

5.2 lid1

5.2 lid1

De ingangsdatum van de vergunning kan niet voor de verzenddatum van de beschikking zijn en zal indien van toepassing aangepast worden. Dit is ook het geval bij een voorgenomen besluit.

7 Concept beschikking voor akkoord CCD

Centrale Commissie Dierproeven

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5.1 lid2h
5.1 lid2e
5.1 lid2h

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Onze referentie
Aanvraagnummer
AVD5.1 lid2h202215867
Bijlagen
3

Datum 5 juli 2022
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte dr. 5.1 lid2e

Op 17 februari 2022 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" met aanvraagnummer AVD5.1 lid2h202215867. 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 uw project uit te voeren binnen de gestelde vergunningsperiode. Deze vergunning wordt afgegeven voor de periode van 5 juli 2022 tot en met 1 maart 2027.

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

Procedure

Advies dierexperimentencommissie
Wij hebben advies gevraagd bij 5.1 lid2h
(hierna: DEC). Dit advies is ontvangen op 30 mei 2022. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, derde lid van de wet.

Nadere vragen aanvrager
Op 31 mei 2022 hebben wij u om aanvullingen gevraagd. U heeft tijdig antwoord gegeven. Het verzoek om aanvullingen had betrekking op het aanpassen van de niet technische samenvatting, het ernstige ongerief in bijlage 2 van de dierproeven en het aantal uur voedseldeprivatie. Uw reactie is betrokken bij de behandeling van uw aanvraag.

Overwegingen
Wij kunnen ons niet geheel vinden in de inhoud van het advies van de DEC. De DEC heeft in haar advies een voorwaarde voorgesteld. De CCD neemt deze voorwaarde niet over omdat de aanvrager zelf in haar aanvraag in bijlage 2 van de dierproeven aangeeft de pilot als go/no-go moment te hanteren. Wel maakt de CCD over het ongerief dat de dieren ondervinden een opmerking in de beschikking.

Aanvullende opmerkingen
U geeft in bijlage 2 van de bijlage dierproeven aan dat u in uw lopende onderzoek AVD5.1 lid2h een pilot uit zal voeren waarin de dosis chemo-en radiotherapie wordt onderzocht. Als blijkt dat ernstig ongerief wordt voorkomen dan zal dit een 'go' moment zijn. Door dit go/no-go moment zal in dit huidige onderzoek geen ernstig ongerief worden verwacht.

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: 0800 789 0789.

Centrale Commissie Dierproeven
namens deze:

5.1 lid2e

drs. F. Braunstahl
Algemeen Secretaris

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

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:
Adres:
Postcode en plaats:
Deelnemersnummer:

5.1 lid2h

deze projectvergunning voor het tijdvak 5 juli 2022 tot en met 1 maart 2027, voor het project "The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease" met aanvraagnummer AVD5.1 lid2h202215867, na advies van 5.1 lid2h. De functie van de verantwoordelijk onderzoeker is 5.1 lid2e Het besluit is gebaseerd op de volgende (aangepaste) stukken:

1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 17 februari 2022
2 de bij het aanvraagformulier behorende bijlagen:
   a Projectvoorstel, zoals ontvangen op 30 mei 2022;
   b Bijlagen dierproeven
      - 3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres, zoals ontvangen op 30 mei 2022;
      - 3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer, zoals ontvangen op 4 juli 2022;
      - 3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases, zoals ontvangen op 30 mei 2022;
   c Niet-technische Samenvatting van het project, zoals ontvangen op 4 juli 2022;
   d Advies van dierexperimentencommissie, zoals ontvangen op 30 mei 2022
   e De aanvullingen op uw aanvraag, zoals ontvangen op 4 juli 2022.

Aanvraagnummer: AVD5.1 lid2h 202215867

Naam proef	Diersoort/ Stam	Aantal dieren	Ongerief
3.4.3.1. Creation and use of primary cell lines, organoids, neurospheres			
	Muizen (Mus musculus)	1.322	100,0% Licht
3.4.3.2. Evaluating the crosstalk between the ENS and other systems on the onset and progression of colorectal cancer			
	Muizen (Mus musculus)	2.118	50,0% Matig 50,0% Licht
3.4.3.3. Evaluating the crosstalk between the ENS and other systems on homeostasis and the onset and progression of inflammatory gut diseases			
	Muizen (Mus musculus)	2.385	35,0% Matig 65,0% Licht

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.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD5.1 lid2h202215867

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.

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.

Van: info@zbo-ccd.nl
Verzonden: vrijdag 29 juli 2022 10:09
Aan: 5.1 lid2h
Onderwerp: Terugkoppeling over projectvergunningsaanvraag AVD5.1 lid2h202215867

Geachte 5.1 lid2h

Op 17-02-2022 hebben wij een aanvraag voor een projectvergunning dierproeven ontvangen waarover uw DEC advies heeft uitgebracht. Het gaat om het project 'The crosstalk between the enteric nervous system, the gut lumen and other tissue environments: from homeostasis to the onset and progression of disease' met aanvraagnummer AVD5.1 lid2h202215867.

De CCD heeft de aanvrager aanvullende vragen gesteld. De aanvullingen hadden betrekking op het aanpassen van de niet technische samenvatting, het ernstige ongerief in bijlage 2 van de dierproeven en het aantal uur voedseldeprivatie.

De CCD heeft besloten de vergunning toe te wijzen. De aanvrager en verantwoordelijk onderzoeker zijn hierover ingelicht. De beschikking is verstuurd op 6-7-2022.

Het DEC advies is helder en navolgbaar. Bij de beantwoording van de beoordelingsvragen verstrekt u een heldere onderbouwing. De CCD had bij vraag C10 graag de mening van de DEC gezien. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen.

Het valt op dat u zeer veel vragen hebt gesteld. Hoewel dit de kwaliteit van de aanvraag verbetert, wekt het ook de suggestie van meeschrijven, wat onwenselijk is. br>
U heeft in uw advies een voorwaarde voorgesteld. Het is de CCD duidelijk waarom u de voorgestelde voorwaarde wilt stellen. Ze neemt deze voorwaarde niet over omdat de aanvrager zelf in haar aanvraag in bijlage 2 van de dierproeven aangeeft de pilot als go- no go moment te hanteren. Er is over dit go-no go moment wel een opmerking in de beschikking opgenomen. br>
De CCD stelt het op prijs dat goed navolgbaar in uw advies is opgenomen dat het advies is gebaseerd op een meerderheidsstandpunt, en waarop het minderheidsstandpunt op gebaseerd is. De CCD had wel graag de onderbouwing gezien wat voor het minderheidsstandpunt doorslaggevend was wat betreft het gebruik van genetisch gemodificeerde muizen. br>
Ook stelt de CCD het op prijs dat bij E3 goed navolgbaar is weergegeven waar discussie over is geweest tijdens de bespreking van deze aanvraag.

Mocht u vragen hebben over onze beslissing, dan kunt u uiteraard contact met ons opnemen.

Met vriendelijke groet,
Namens de Centrale Commissie Dierproeven

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