nr.	**Inventaris Woo W24-01**	**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
nr.	**NTS 202317174**									
1	Aanvraag d.d. 4 juli 2023				x		x		x	
2	Project voorstel bij aanvraag				x		x		x	
3	Bijlage dierproeven bij aanvraag				x		x		x	
4	DEC advies d.d. 2 november 2023				x		x		x	
5	Projectaanvraag gewijzigd na DEC advies				x		x		x	
6	Projectvoorstel gewijzigd na DEC advies				x		x		x	
7	Bijlage dierproef gewijzigd na DEC advies				x		x		x	
8	Mail CCD nadere vragen VGH d.d. 10 november 2023				x		x		x	
9	Projectvoorstel gewijzigd na vragen CCD				x		x		x	
10	Bijlage dierproef gewijzigd na vragen CCD				x		x		x	
11	NTS gepubliceerd			x						
12	Adviesnota aan CCD d.d. 22 november 2023				x		x		x	x
13	Beslissing op aanvraag d.d. 7 december 2023				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
	5.1 lid2e			
E-mailadres gemachtigde	5.1 lid2e			

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	5.1 lid2e	
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		
	E-mailadres		

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 - 09 - 2023
	Einddatum (t/m)	31 - 08 - 2028
3.2 Wat is de titel van het project?	Unravelling the underlying mechanisms of early life stress induced disorders in animals with reduced expression of the serotonin transporter	
3.3 Wat is de titel van de niet-technische samenvatting?	Het definiëren van een nieuw model voor kwetsbaarheid voor depressie	
3.4	Naam DEC	5.1 lid2h
	Postadres	5.1 lid2h

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 1
☒ 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	5.1 lid2e
Plaats	5.1 lid2h
Datum	27 - 6 - 2023
Handtekening	5.1 lid2e

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0800-7890789).

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.	Transgenerational susceptibility to depression due to early life stress

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.

Depression is a mental health condition characterised by sadness, loss of interest or pleasure, feelings of guilt and low self-worth that substantially impairs an individual's ability to cope with daily life. Cognitive

impairments, altered affective behaviour, and social disfunctions are often present in depressed individuals [1-3]. Depression is the most prevalent neuropsychiatric disorder and the first cause of disability in the world [4]. The social burden related to depression is steadily increasing, and the numbers for depressive disorders may increase even more in the next few years due to the unprecedented negative effects of the COVID-19 pandemic on mental health.

Women suffer from depression twice as often compared to men [5] and unfortunately pregnant women are not spared. Approximately one out of five pregnant women suffer from depressive symptoms [6], which can have long-term health consequences in the offspring [7]. Early life programming through an adverse intrauterine environment, or postnatally, increases susceptibility to a myriad of diseases, including psychiatric disorders [8]. Thus, an adverse early life environment increases the risk for depression. However, this risk is increased when a subject has vulnerable genes as was shown by the seminal paper of Caspi et al. [9]. This, and later studies identified the critical interaction of genetic and environmental factors that contribute to depression [10-13]. Caspi and colleagues found that the risk for depression increased when the number of stressful life events that people encountered increased. Additionally, they studied the extent to which the genotype that regulates the serotonin transporter (SERT) expression moderates the influence of stress on depression. In the serotonin-transporter-linked promoter region (5-HTTLPR), different lengths of the repetitive sequence containing GC-rich, 20-23-bp-long repeat elements in the upstream regulatory region of the gene have been identified. Deletion or insertion in the 5-HTTLPR is referred to as the 14-repeat short (S, low expressing) and the 16-repeat long (L, high expressing) alleles. Caspi and colleagues found that individuals who were carriers of one or two copies of the S-allele were more vulnerable to developing depression following stress exposure than carriers of the L-allele exposed to stress [9].

Many studies reproduced the Caspi's research, although a large genome-wide study found no association between the SERT genotype and increased risk for lifetime prevalence of depression in people exposed to stress [14]. Also, rodent studies fail to show solid evidence for increased vulnerability to developing depressive-like behaviour after early-life stress (ELS) in rodents with reduced SERT (heterozygous; $SERT^{+/-}$) expression [15]. Therefore, the potential association of the SERT gene and psychiatric condition remains inconclusive.

What has been consistently shown is that ELS exposure has a large impact on mental health later in life [16,17], and that ELS is not restricted to stress exposure during childhood (postnatal stress), but can also include exposure to stress during the foetal period, mediated by a mother that is stressed during pregnancy or even before conception [18,19]. Thus, ELS to the offspring can take place by 1) maternal stress/depression before pregnancy (*pregestational*; which is transferred to the offspring during pregnancy); 2) maternal stress/depression during pregnancy (*prenatal*; transferred to the offspring during pregnancy); and 3) either maternal stress/depression, or direct stressors to the offspring after birth (*postnatal*; during the first years of life).

With this additional evidence of maternal stress mediating offspring long-term outcomes, the studies about the ELS x SERT genotype interaction can be revisited. One of the reasons that the ELS x SERT studies are inconclusive may be because sustained maternal stress, starting before child's birth and even before conception, has not been taken into account. Moreover, clinical research supports the notion that neuropsychiatric disorders, including depression, have a developmental origin mediated by the conditions of the mother before the conception of the child [18]. From this view, the exposure to *cumulative* ELS can interact with the SERT genotype -of the offspring- to increase the risk of depression. To our knowledge, no one has studied whether the onset of depression in the adult offspring is mediated by the interaction of *cumulative* ELS exposure and the SERT genotype. The study published by Tiemeier et al. [20] strongly suggest the importance to do so. They found that maternal anxiety -that involves stress in the mother- during pregnancy and postnatally increased the risk of child emotional problems and leads to less accurate emotional matching in 3-year-old S-allele carriers.

There are ethical and methodological limitations to control the timeframe and type of *cumulative* ELS exposure that leads to elucidate its role in the adult onset of depression in S-allele carriers. Fortunately,

valid animal models can help to study this association. Rodents do not express the 5-HTTLPR; however, heterozygous SERT knockout rodents ($SERT^{+/-}$) show neurochemical similarities to human S-allele carriers [21,22]. From a previous research project of our group, we provided a comprehensive overview of the behavioural effects of ELS in $SERT^{+/-}$ rodents and the neurobiological mechanisms involved [15]. We found that studies of postnatal ELS in $SERT^{+/-}$ rodents failed to show solid evidence for increased vulnerability to a depressive-like phenotype when $SERT^{+/-}$ adult animals were tested. We also performed a study in $SERT^{+/-}$ female rats to test whether their exposure to postnatal ELS increased their vulnerability to depressive-like behaviours when they were adults. The average of $SERT^{+/-}$ females did not exhibit consistent behavioural changes of depressive-like responses after ELS, although some females exposed to postnatal ELS showed increased affective-related behaviours [23].

Considering the findings of our previous research project, postnatal ELS is not optimal or severe enough to induce a depressive-like phenotype in $SERT^{+/-}$ rodent models. In the present research project, we will investigate whether $SERT^{+/-}$ male and female rats are more vulnerable to develop a depressive-like phenotype in adulthood following the exposure to *cumulative* ELS. Moreover, this project will help us understand the underlying mechanisms of SERT x *cumulative* ELS interactions in the onset of depression later in life.

Based on our findings and the literature, we propose to apply pre-gestational, prenatal and postnatal stressors and investigate how these stressors cumulatively affect wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ offspring (both male and female). Not only are we interested in the mechanisms underlying this multiple stressor system, but also whether the SERT gene expression level increases the vulnerability to depression following *cumulative* ELS. This will prevent using a high number of animals in the future as we could select more animals expressing a depressive-like phenotype after a single manipulation of multiple ELS stressors and use this for instance as a model for maternal stress.

We will study cognitive, affective-related and social behaviours, as well as gene expression and epigenetic markers (study 1). We will also characterise the social functioning and neurobiological mechanisms involved in studies 2 to 4. Lastly, we will test whether the offspring of the depression-like parents also express a depressive-like phenotype, as well as the extent to which epigenetic markers are shared in both generations (study 5).

In summary, it is critical to get fundamental insight in how SERT and ELS interact to induce stress-related disorders, not only to understand the mechanisms underlying these vulnerabilities, but also to pave the way for further research into new treatment targets of (maternal) depression. Because of the mechanistic approach in this research this is considered basic research. We hypothesise that a cumulative history of ELS exposure will increase the risk of disrupted cognitive, affective-related, social behaviours in the offspring. In addition, we hypothesise that animals with a vulnerable genetic background ($SERT^{+/-}$ rats) will be more vulnerable to develop depressive-like behaviours (or in a more severe fashion) than those with a non-vulnerable genetic background. Finally, we hypothesise that the effects of stressful early-life experiences of parents will be transferred to their offspring through inheritance of epigenetic markers.

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 research is to determine whether the interaction of *cumulative* ELS and the SERT genotype increases the vulnerability to developing a depressive-like phenotype and to what extent the parental depressive-like phenotype is transferred to the next generation. Neurobiological mechanisms -including epigenetic markers-, depressive-like behaviours -including affective-related, cognitive and social behaviour- and transgenerational effects will be elucidated in the rat model.

To reach this ultimate goal, immediate goals are proposed as follows:

Goal 1: To determine whether *cumulative* ELS induces a depressive-like phenotype in the rat.
Goal 2: To assess neurobiological mechanisms involved in the depressive-like phenotype induced by *cumulative* ELS.
Goal 3: To determine whether the $SERT^{+/-}$ genotype increases the risk for depressive-like behaviour after *cumulative* ELS and evaluate the neurobiological mechanisms involved.

Goals 1 to 3 will be attained by conducting study 1.

Goal 4: To identify whether social functioning and its neurobiological mechanisms are altered in young and adult rats exposed to *cumulative* ELS.

Goal 4 will be attained by conducting studies 2 to 4.

Goal 5: To determine the extent to which the depression-like phenotype induced by *cumulative* ELS in one generation is transferred to the next and evaluate the neurobiological mechanisms involved.

Goal 5 will be attained by conducting study 5.

3.2.2 Provide a justification for the project's feasibility.

Our research group has ample experience conducting behavioural experimentation to assess cognition, affective-related and social behaviours in rodents, especially also in serotonin transporter knockout animals (SERT). The SERT animals have been bred at our facility for many years, making the project feasible. In addition, our research group has experience in performing molecular analyses (microarray, qPCR, genotyping, DNA methylation, DNA hydroxymethylation, histone methylation).

We do not expect technical difficulties in conducting the experiments as we already have experience in studying ELS effects on brain development and behaviour of transgenic models.

All equipment and housing needed for experiments are available at the institute to which our research group belongs.

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?

This project addresses the influence of an environmental factor interacting with a genetic factor in the development of stress-related disorders. It also provides mechanistic insights in the effects of *cumulative* ELS on the development of stress-related disorders, especially in relation to the serotonin transporter genotype. Good animal models are indispensable in this research, as we can invasively study the underlying mechanisms in the brain of animals exposed to ELS mediated by the maternal stress (pregestational and prenatal), something that is not possible in humans.

If the outcome shows that more rats express a depressive-like phenotype as a result of *cumulative* ELS, we will be able to elucidate the underlying mechanisms contributing to stress-induced disorders. In addition, if $SERT^{+/-}$ rats exhibit higher vulnerability to developing a depressive-like phenotype following the *cumulative* ELS exposure, we will be able to demonstrate that the serotonin transporter genotype

moderates the influence of stress on depression. This would greatly benefit the clinical management of stress-induced disorders as new targets for drug treatment may be revealed.

Additionally, if altered social behaviours are observed in adult and especially also in young animals exposed to *cumulative* ELS, we will be able to show a different neurodevelopmental trajectory of social functioning induced by *cumulative* ELS presumably related to the late onset of depression.

Lastly, by studying if the parental depressive-like phenotype induced by *cumulative* ELS is transferred to the next generation, we will provide evidence of epigenetic signatures of the depressive-like phenotype shared between parents and the offspring.

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

Our research group along with other researchers conducting basic research on stress-related disorders, including depression, can use this animal model to further investigate neurobiological underpinnings related to how *cumulative* ELS influences the development of the brain to induce depressive-like behaviours, both separately and in combination with the SERT genotype. This model would also allow further investigation of the mechanisms underlying transgenerational effects of depression and the onset of social dysfunctions related to depression.

In the long term, pharmaceutical companies focused on developing pharmacological agents may be interested in this animal model to test the efficacy of different drugs in treating depressive-related symptoms.

As experimental subjects, the condition of rats as stakeholders is done under the principles of the 3Rs and setting go/no-go moments. The refinement of depression models (cumulative ELS and/or SERT genotype) can lead to a decrease in the number of animals used for this purpose in the future.

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.

Study 1: the depressive-like phenotype by *cumulative* ELS
We will expose wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ rats to *cumulative* ELS to test whether they develop depressive-like behaviours when they are adults.

For this research project *cumulative* ELS refers to the combination of (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS exposure (see appendix 1 to this form for further description of each ELS type).

Four conditions, one control and three experimental, will be compared to establish whether *cumulative* ELS induces depressive-like responses in a robust fashion:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition 1: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to postnatal ELS (ELS treatment 1).
Experimental condition 2: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) prenatal ELS and postnatal ELS (ELS treatment 2).
Experimental condition 3: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS (ELS treatment 3).

We will test affective-related behaviour, cognitive performance and social functioning in adult $SERT^{+/+}$ and $SERT^{+/-}$ rats as indicatives of depressive-like behaviours. We will also analyse mRNA expression level of selected genes, and epigenetic markers of those genes, in brain regions that are known to be involved in depression. Selected genes will at least include those involved in the serotonergic system, brain stress system (HPA axis), myelination and neural growth and survival as many of them can be co-expressed in major neural circuit connections involved in depressive-like behaviours in rodents [24]. See appendix 1

to this form, section A for detailed description of the general design of the animal procedures and molecular analyses.

The milestone of this study will be finding significant differences in behavioural outcomes, the expression level of genes, and epigenetic markers between $SERT^{+/+}$ and $SERT^{+/-}$ animals exposed to ELS treatments and controls.

Selection points and decision criteria
The experimental design and execution of studies 2 to 5 will be based on how the questions in the black squares below are solved according to findings in study 1:

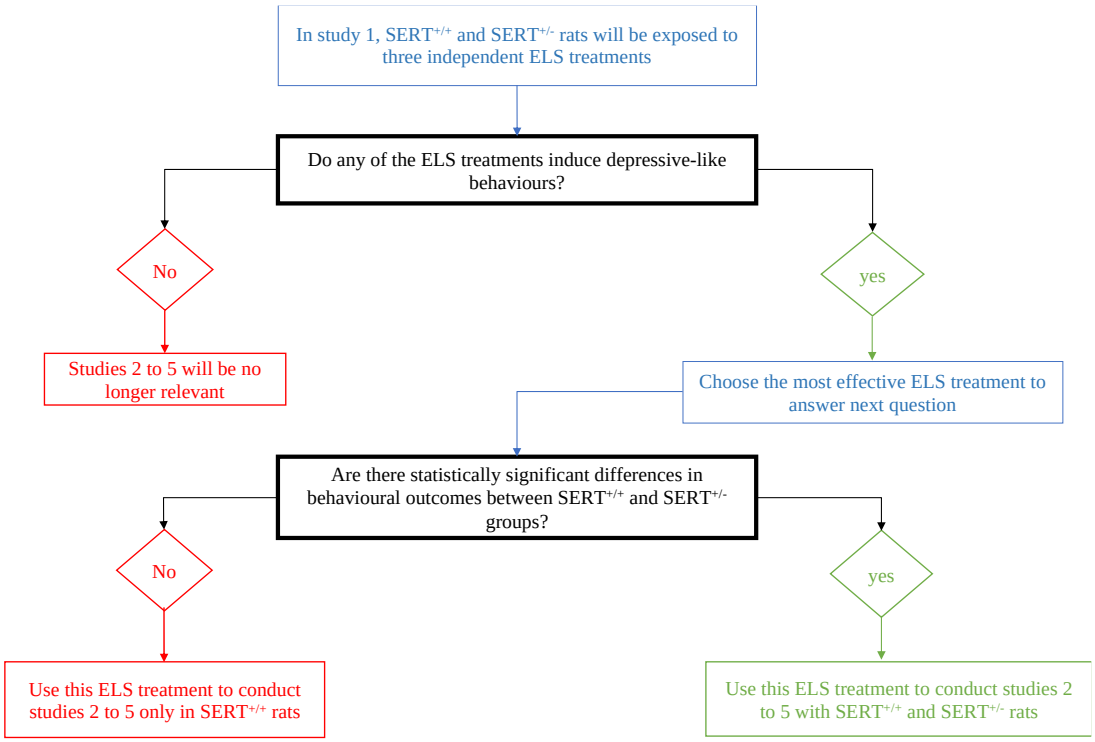

Fig1. Decision criteria to conduct the following studies based on findings of study 1. ELS treatment 1: postnatal ELS exposure; ELS treatment 2: (maternal) prenatal ELS and postnatal ELS; ELS treatment 3: (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS.

If depressive-like behaviours are not expressed in animals due to any of the ELS treatments in comparison to controls in study 1, the following experiments will no longer be relevant. Otherwise, we will select the ELS treatment that most robustly induces the depressive-like phenotype to use in the following studies. Consequently, we will discard the other ELS treatments. In addition, if $SERT^{+/-}$ rats do not express more vulnerability to depressive-like behaviours than $SERT^{+/+}$ rats, then $SERT^{+/-}$ rats will no longer be used in studies 2 to 5, and only $SERT^{+/+}$ animals will be included.

Study 2: focus on offspring social functioning in the juvenile period
We will expose $SERT^{+/+}$ and (depending on study1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. Next, we will compare social play behaviour between them when they are juveniles (both males and females). After the social play test, we will collect the brain tissue to analyse neuronal activity - through c-Fos expression by immunohistochemistry- in brain regions that regulate social play behaviour (e.g., prefrontal cortex, dorsal and ventral striatum, amygdala, habenula; [25]). See appendix 1 to this form for detailed description of the general design of the animal procedures.

The milestone of this experiment will be finding differences in social play behaviour and c-Fos expression in brain regions that regulate this behaviour between animals exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Study 3: focus on offspring social functioning in adulthood
We will expose $SERT^{+/+}$ and (depending on study1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. When animals are adults, we will assess sexual behaviour and aggressive behaviour in both males and females. After the last aggression test, we will collect the brain tissue to analyse neuronal activity -through c-Fos expression by immunohistochemistry- in regions of the social brain network that regulate this behaviour (e.g., medial preoptical area, periaqueductal grey matter, ventromedial hypothalamus, medial amygdala, [26]). See appendix 1 to this form for detailed description of the general design of the animal procedures.

Encountering intruders in their own territory induce a stress response in male and female rodents [27]. One way to measure this response is by assessing corticosterone (the main stress hormone in rodents) after the rat encounters an intruder in his/her territory [27,28]. Hence, when we test aggressive behaviour, we will take the aggression test as an opportunity to evaluate if corticosterone levels are differently affected by ELS treatment, SERT genotype, or its combination when the rat encounters an intruder in his/her own home cage.

Milestone #1 will be finding significant differences in sexual and aggressive behaviours between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Milestone #2 will be finding significant differences in c-Fos expression, after the aggression test, in brain areas that regulate aggressive behaviour between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Milestone #3 will be finding significant differences in corticosterone levels following the aggression test between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Note: detailed procedures to test social play, sexual and aggressive behaviours and c-Fos expression in brain regions related to these behaviours are described in the appendix 1 of this proposal, section A, studies 2 and 3, respectively. Supported literature is provided correspondingly.

Study 4: unravelling molecular mechanisms of social functioning
We will evaluate the mRNA expression for selected genes, and epigenetic markers of those genes, in brain regions of the social brain network [26]. We will expose $SERT^{+/+}$ and (depending on study 1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. Next, animals will be left undisturbed until they are ±24 weeks old, when we will collect their brain tissue. This time of brain tissue collection is selected to be similar to the time in which we will collect the brain tissue in study 3. Selected genes will at least include those that code for vasopressin, serotonin, corticoid and oxytocin receptors as many of them can be co-expressed in several regions of the social brain network. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures.

The milestone of this experiment will be finding significant differences in mRNA expression and epigenetic markers of brain regions comprising the social brain network between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
We will determine if the depressive-like phenotype in one generation is transferable to their offspring. To accomplish this, first we will induce a depressive-like phenotype by exposing $SERT^{+/+}$ and $SERT^{+/-}$ rats to

the ELS treatment that most robustly induced depressive-like behaviours in study 1. ($SERT^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, only $SERT^{+/+}$ rats will be used).

When F1 are adults, we will test their affective-related behaviour to confirm that the depressive-like phenotype is expressed. Then, depressed-like F1 males and depressed-like F1 females will be mated with wildtype, control females and males, respectively. The offspring of F1 will be referred as F2.

After delivery of F2, social communication of F2 pups and maternal care of F1 females during the first postnatal week of F2 will be tested. See appendix 1 to this form, section A for further explanation of these outcomes as indicatives of an altered behavioural phenotype.

To test whether the depressive-like phenotype is expressed in F2, affective-related, cognitive, and social behaviours, gene expression and epigenetic markers of F2 will be assessed. Selected genes for mRNA expression analysis and measurement of epigenetic markers will be the same as for the study 1. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures.

Milestone #1 will be finding significant differences in affective-related, cognitive and social behaviours between F2 born from depressed-like parents and F2 born from non-depressed-like parents.

Milestone #2 will be finding significant differences in maternal care behaviour between depressed-like and non-depressed-like F1 female rats.

Milestone #3 will be finding significant differences in gene expression of selected genes and epigenetic markers between F2 born from depressed-like parents and F2 born from non-depressed-like parents.

Milestone #4 will be finding similar epigenetic changes between depressed-like F1 and depressed-like F2.

Coherence: As we seek to investigate whether the cumulative ELS induces depressive-like behaviours, and to determine whether $SERT^{+/-}$ rats are more vulnerable than wildtypes, the comparison between the three ELS treatments and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ should be the first step. By selecting the most robust ELS treatment to induce the depressive-like phenotype, the further study of the social functioning in depression, its neurobiological mechanisms, and transgenerational effects will be carried out by conducting studies 2 to 5.

3.4.2 Provide a justification for the strategy described above.

All studies proposed above are based on the principles of experimental research. For each study we have:

1. Established hypothesis to be empirically tested
2. Defined independent variables to be manipulated
3. Defined dependent variables to be measured
4. Followed experimental designs to establish relevant groups of comparison and control unknown and/or confounding variables

By following this strategy, we seek to establish a causal relationship between variables. We expect to demonstrate that the occurrence of depressive-like behaviours, altered gene expression and distinctive epigenetic markers of relevant genes (dependent variables) arise due to *cumulative* ELS exposure alone or in combination with the SERT genotype (independent variables).

The order in which the studies will be performed is important. In study 1 we will test if *cumulative* ELS induces a depressive-like phenotype in a robust fashion, alters gene expression and changes epigenetic signatures. We will only perform the following studies if a significant effect is found in this study. In studies 2 to 4 we will further characterise the social functioning and neurobiological mechanisms involved. Finally, we will investigate if the offspring (F2) of depressive-like parents (F1) also express a

depressive-like phenotype, exhibit altered mRNA expression, and manifest changes in epigenetic markers.

By conducting these studies, we will answer the question whether the interaction of *cumulative* ELS and the SERT genotype increases the vulnerability to develop a depressive-like phenotype and provide mechanistic insights in stress-related disorders. In addition, we will understand how the neurodevelopmental trajectory of social functioning in depression, induced by ELS, is characterised. Finally, we will know to what extent the parental depressive-like phenotype is transferred to the next generation.

The selected strategy will allow us to optimise the use of animals and reduce their number where possible. The study 1 will be critical in this respect. Only the ELS treatment that produces the most robust depressive-like phenotype will be used in the follow-up studies. In addition, if no clear SERT genotype effect is found, the $SERT^{+/-}$ animal will not be used in the following studies.

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	Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.

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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 (0800-7890789).

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: Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter

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.

Study 1: the depressive-like phenotype by *cumulative* early life stress (ELS)

We will test whether *cumulative* ELS induces a depressive-like phenotype, and whether SERT$^{+/-}$ rats are more vulnerable compared with SERT$^{+/+}$ rats. To accomplish this, SERT$^{+/+}$ and SERT$^{+/-}$ rats will be exposed to *cumulative* ELS.

For this research project *cumulative* ELS refers to the combination of (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS exposure. See the next section for detailed procedure of each type of ELS.

From 14 weeks of life, SERT$^{+/+}$ and SERT$^{+/-}$ rats will be assessed for affective-related, cognitive and social behaviours. For this assessment, we will use the behavioural tests indicated below:

1- Elevated plus maze (EPM), sucrose preference test (SPT), and open field test (OFT) to evaluate affective-related behaviours.
2- Object location test (OLT) and novel object recognition (NOR) to test cognitive performance.
3- Social interaction test and social recognition test to assess social investigation, social memory and social withdrawal.

The tests will be conducted at least one week apart in the following order: EPM, SPT, OFT, OLT, NOR, social interaction test, and social recognition test. See next section about description of proposed animal procedures of study 1, for detailed procedure of each behavioural test.

After the last behavioural test (±20 weeks), animals will be sacrificed by fast decapitation and their brain tissue will be collected to analyse gene expression of selected genes, and epigenetic markers of those genes, in brain regions that are known to be involved in depression. Selected genes will at least include those involved in the serotonergic system, brain stress system (HPA axis), myelination, neural growth and survival as many of them can be co-expressed in major neural circuit connections involved in depressive-like behaviours in the rat (e.g., prefrontal cortex, hippocampus, amygdala) [1].

The chronology of this study is summarised in fig1:

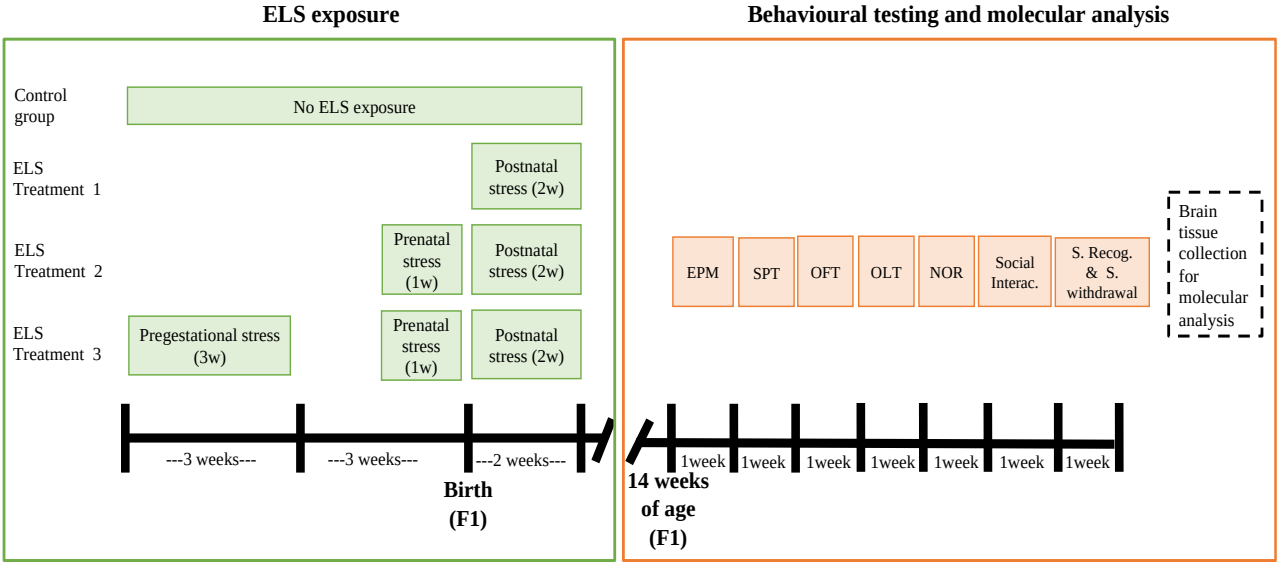

Fig1. General design of study 1

-The EPM is useful test to evaluate anxiety-related behaviours. It is based on the natural tendency of rodents to avoid open spaces and stay in enclosed spaces. The more exploration in open arms, the more indication of reduced levels of anxiety.
-The SPT aims to evaluate the capacity of an animal to experience pleasure by consuming palatable food. It is based on the natural preference of rodents for sweets. Low levels of sucrose consumption are indicative of anhedonia, a central symptom in depressive disorders
-The OFT aims to evaluate anxiety-like behaviours. It is based on the natural tendency of rodents to avoid open spaces and exhibit thigmotaxis. The more time the animal spends walking along the walls or staying in the corners of the arena, the higher the index of anxiety-like responses.
-The OLT is useful to evaluate spatial short-term and long-term memory in rats. It is based on the spontaneous tendency of rodents, previously exposed to two identical objects, to later explore one of the objects—placed in a novel location—for a longer time than they explore the non-displaced object. The more time the animal spends with the object in the new location, the higher the level of spatial memory.
-The NOR aims to evaluate short and long-term recognition memory. It is based on rodents' natural tendency to explore novel features in their environment, including objects. The more time the animal spends with the new object, the higher the level of recognition
-Social interaction test, performed in an open arena, aims to evaluate investigation towards new social stimuli. It is based on the natural tendency of rodents to investigate unfamiliar mates. Several behaviours such as sniffing, grooming, or mounting can be measured as an index of social interaction.
-Social recognition test aims to assess the ability to recognise a novel mate in comparison to familiar mates. This test is based on the natural tendency of rodents to investigate unfamiliar partners. By performing this test in a three-chamber box, the more time spent in the box with the novel partner, the higher the index of social discrimination. An additional benefit of using this apparatus is the possibility to evaluate social withdrawal, i.e., the lack of motivation to have social contact. Social withdrawal can be assessed by measuring the time spent in the box in which neither the familiar nor the unfamiliar mate are present.

Four conditions, one control and three experimental, will be compared to establish whether *cumulative* ELS induces depressive-like responses in a robust fashion:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition 1: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to postnatal ELS (ELS treatment 1).
Experimental condition 2: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) prenatal ELS and postnatal ELS (ELS treatment 2).

Experimental condition 3: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS (ELS treatment 3).

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: SERT genotype; Factor 3: Sex.

The primary outcome parameters to test the onset of depressive-like behaviours induced by *cumulative* ELS will be the responses displayed by the rats in the behavioural tests mentioned above (also shown in orange boxes of fig1). The selection of these tests is based on previous research conducted in our research group and elsewhere [2-4] showing their validity to assess several parameters of affective-related behaviour, cognition, and social behaviours in rodents.

The primary outcome parameter to test the changes of gene expression in the brain regions related to depressive-like behaviours induced by *cumulative* ELS will be the level of mRNA expressed for selected genes. The selection of mRNA gene expression analysis is based on previous evidence indicating that lower levels of mRNA expression of genes related to serotonergic system, brain stress system and neural growth correlated with maladaptive responses that increases the risk for depression [5]. Quantification of mRNA expression will be done by using the quantitative polymerase chain reaction (RT-qPCR) technique.

The primary outcome parameter to test epigenetic markers induced by *cumulative* ELS will be the level of DNA methylation at the promotor regions of selected genes. The DNA methylation analysis is selected because changes in DNA methylation (in general, hypermethylation) have been associated with depression [6]. Measurement of DNA methylation status in promotor regions of selected genes will be performed by PCR amplification of bisulfite-converted DNA [7].

It should be noted that additional ELS treatments without postnatal ELS are not relevant to this research project. If we exclude this period, the model will not resemble an important aspect of the human condition we seek to translate into the animal, i.e., adversity during the first years of life as a risk factor for depression later in life.

Studies 2 to 5 will be performed *only* if a depressive-like behavioural phenotype is found in this study. See section 3.4.1 of the project proposal form to see the explanation of selection points and decision criteria in this respect.

Study 2: focus on offspring social functioning in the juvenile period
Here we will test whether *cumulative* ELS alters social functioning in the juvenile period, and whether $SERT^{+/-}$ rats are more vulnerable compared with $SERT^{+/+}$ rats (depending on study1). To accomplish this, $SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

At ±4-5 weeks of age, animals will be assessed for social play behaviour in the social play-fighting test. This test will take place for 20 minutes (see next section for detailed procedure of social play-fighting test). After 90-120 minutes the social play-fighting test is over, animals will be sacrificed and brain tissue will be collected by perfusion to analyse the c-Fos protein expression level in brain regions that regulate social play behaviour (e.g., prefrontal cortex, dorsal and ventral striatum, amygdala, habenula) [8].

The chronology of this study is summarised in fig2:

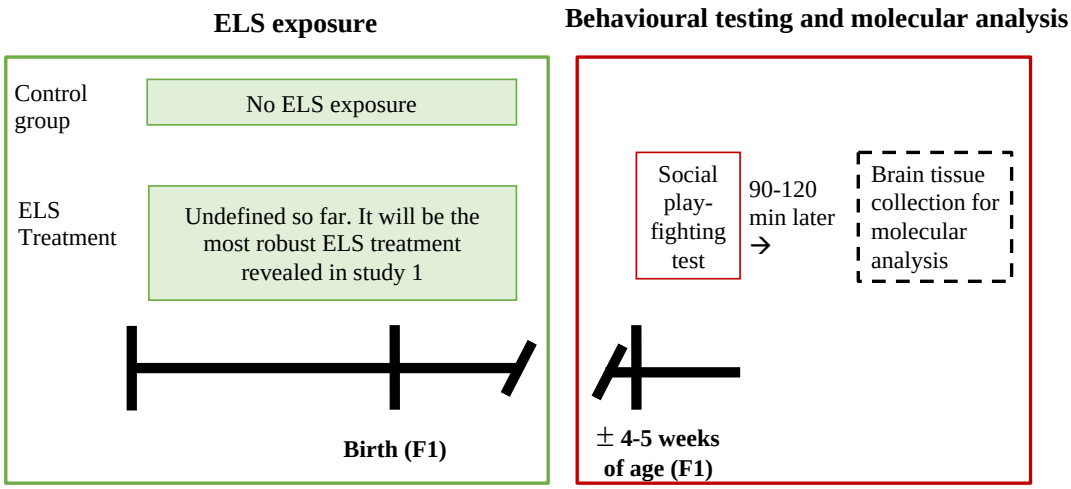

Fig2. General design of study 2

Two conditions, one control and one experimental, will be compared:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.
Experimental condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if SERT$^{+/-}$ animals are included).

The primary outcome parameters to test the changes in social play behaviour induced by *cumulative* ELS will be the responses displayed by the rats in the social play-fighting test. This test is selected because it allows to assess behavioural patterns of social play behaviour that are highly expressed in rodents at 35-42 days of age [9].

The primary outcome parameter to test changes in neuronal activity of brain regions that regulate social play behaviour induced by *cumulative* ELS will be the level of c-Fos protein expression in neurons of such brain regions. This expression level will be quantified through c-FOS protein immunohistochemistry staining to count c-Fos-positive neurons. c-Fos protein is expressed rapidly and transiently after external stimuli [10]. Therefore, the c-Fos protein expression level will serve as an indirect biomarker of neuronal activity in brain areas that regulate social play behaviour during the social play-fighting test.

Study 3: focus on offspring social functioning in adulthood
Here we will test whether *cumulative* ELS alters social functioning in adulthood, and whether SERT$^{+/-}$ rats are more vulnerable (depending on study1). To accomplish this, SERT$^{+/+}$ and SERT$^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

From 14 weeks of age, sexual behaviour of males and females will be tested once a week for 6-10 consecutive weeks. In order to prevent pregnancy in females during the sexual behaviour testing, a double tubal ligation surgery will be performed two weeks before the behavioural testing begins. See next section about description of proposed animal procedures of study 3, for details of this surgery procedure.

To test sexual behaviour in males, we will place the rat with a stimulus female in a testing cage. To test sexual behaviour in females, we will place the rat with a stimulus male in a testing cage. After the last sexual test has been taken place, males will be housed individually with a companion female in a new home cage to elicit territorial behaviour. Likewise, females will be housed individually in a new home cage to elicit territorial behaviour. One week later, we will use the resident-intruder test in males, and the female-intruder test in females to test aggressive behaviour. See next section about description of proposed animal procedures of study 3, for detailed procedure of each behavioural test.

As aggressive encounters induce a stress response in animals, we evaluate the stress response induced by the resident-intruder test in males and the female-intruder test in females. We will collect three blood samples from the animals' tail to assess the concentration level of corticosterone (the main stress hormone in rodents) in systemic circulation. We will collect three blood samples in total (one before, and two after the aggression test) because this will help us to track the changes in the corticosterone level as a result of being exposed to a

stress challenge (i.e., the aggression test). See next section about description of proposed animal procedures of study 3, for detailed procedure of blood sampling.

After the resident-intruder test and collection of blood samples (90-120 minutes), animals will be sacrificed, and the brain tissue will be collected by perfusion to analyse the c-Fos expression level in brain regions that regulate aggressive behaviour [11].

The chronology of this study is summarised in fig3:

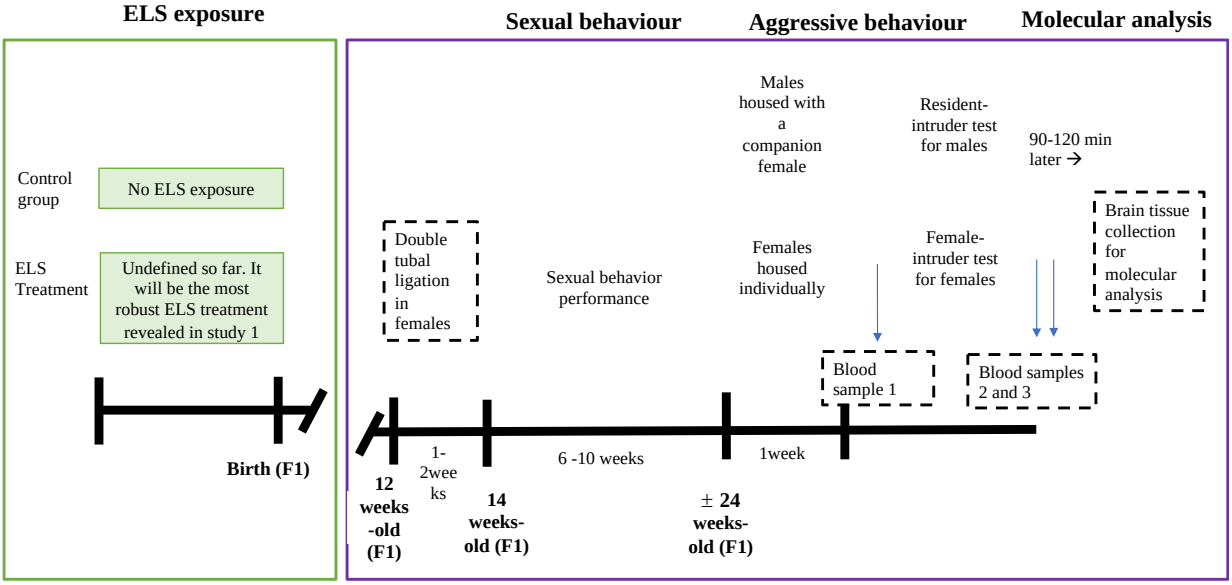

Fig3. General design of study 3. The symbol ± means approximately. It is possible that sexual behaviour testing takes fewer than 10 weeks. Therefore, the individual housing of males and females, the aggression test and the brain collection may take place few weeks earlier.

Two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if $SERT^{+/-}$ animals are included).

Readouts of sexual behaviour test, resident-intruder test, and female-intruder test will be the primary outcome parameters to test the changes in adult social behaviour induced by *cumulative* ELS. Sexual behaviour testing is selected because adult males express copulatory behaviours in the presence of females, and females express proceptive and receptive behaviours (mediated by the oestrus cycle) in the presence of males. The resident-intruder test for males and the female-intruder test for females are selected because adult rodents express territorial aggression when encountering intruders in their own territory.

Systemic concentration level of corticosterone will be the primary outcome parameter to test whether *cumulative* ELS alters the stress response in adult rats following the exposure to a stress challenge (i.e., the aggression test). Blood sampling is selected because corticosterone is released from adrenal glands into blood circulation.

c-Fos-positive neurons expressed in brain regions that regulate aggressive behaviour will be the primary outcome parameter to test changes in neuronal activity induced by *cumulative* ELS. As mentioned above, c-Fos protein is expressed rapidly and transiently after external stimuli [10]. Therefore, the c-Fos protein expression level will serve as an indirect biomarker of neuronal activity in brain areas that regulate the aggressive behaviour during the aggression test.

Study 4: unravelling molecular mechanisms of social functioning
Here we will test the underlying molecular mechanisms related to the social brain network in adult animals exposed to *cumulative* ELS, and whether $SERT^{+/-}$ rats exhibit a different molecular pattern in comparison to $SERT^{+/+}$ (depending on study 1). To accomplish this, $SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in

study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

The animals will be left undisturbed (except for cage cleaning) until ±24 week of age in which they will be sacrificed by decapitation to collect the brain tissue. We will analyse the mRNA expression of selected genes in brain regions of the social brain network. We will also test the DNA methylation level at the promotors of those selected genes. Selected genes will at least include those that code for vasopressin, serotonin, corticoid and oxytocin receptors as many of them can be co-expressed in several regions of the social brain network.

The chronology of this study is summarised in fig4:

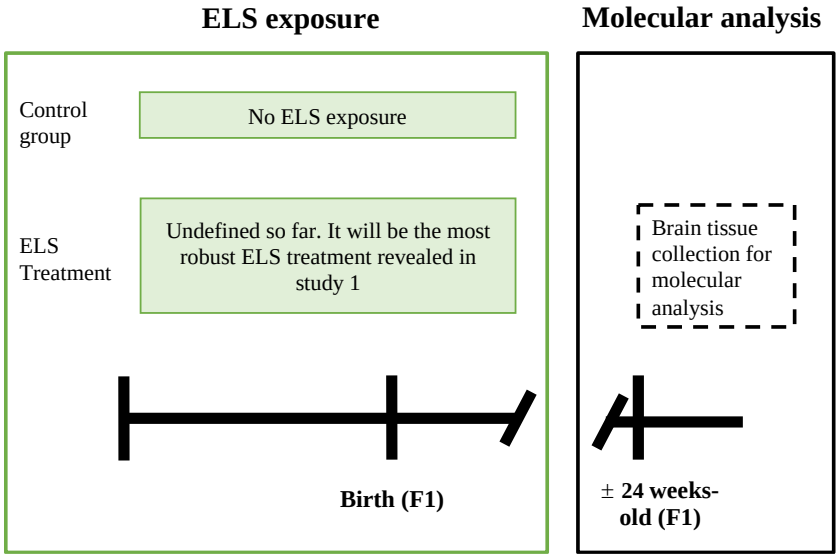

Fig4. General design of study 4. The symbol ± means approximately. This time point may vary depending on the time in which brain tissue will be collected in study 3.

Two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if $SERT^{+/-}$ animals are included).

No behavioural procedures will be performed before brain collection to prevent changes in gene expression and epigenetic markers as a result of behavioural tests. We will collect brains at ±24 week of age to make these results comparable to findings in study 3.

The primary outcome parameter to test the changes of gene expression in brain regions of the social brain network induced by *cumulative* ELS will be the level of mRNA expressed for selected genes. The selection of mRNA gene expression analysis is based on previous evidence indicating that gene expression of selected genes in brain regions comprising the social brain network are closely linked to expression of social behaviours like aggression [12]. Quantification of mRNA expression will be done by using the quantitative polymerase chain reaction (RT-qPCR) technique.

The primary outcome parameter to test epigenetic markers induced by *cumulative* ELS will be the level of DNA methylation at the promotor regions of selected genes in brain regions of the social brain network. The DNA methylation analysis is selected because changes in DNA methylation (in general, hypermethylation) have been associated with vulnerability to abnormal social functioning [13]. Measurement of DNA methylation status in promotor regions of selected genes will be performed by PCR amplification of bisulfite-converted DNA [7].

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
Here, we will determine if the depressive-like phenotype in generation F1 is transferable to their offspring F2. To accomplish this, first we will induce a depressive-like phenotype in F1 by exposing $SERT^{+/+}$ and $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. ($SERT^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, only $SERT^{+/+}$ rats exposed to ELS will be used).

When F1 males and females are adults, we will test affective-related behaviours to confirm that the depressive-like phenotype is expressed. To accomplish this, from 14 weeks of age (similar to study 1), affective-like behaviours of F1 will be tested by using the same tests used in study 1 (i.e., EPM, SPT and OFT). The tests will be conducted at least one week apart in the following order: EPM, SPT, OFT. After the last behavioural test, F1 animals will be mated to create F2. To do so, depressed-like males and depressed-like females will be mated with wildtype, control females and males, respectively.

F1 pregnant females will be left undisturbed during pregnancy (except for cage cleaning). After delivery of F2, we will measure social communication of F2 pups on postnatal day 6 and maternal care of F1 females during the first postnatal week of F2. See next section about description of proposed animal procedures of study 5, for detailed procedure of these behavioural tests.

When F2 are 14 weeks old (similar to study 1), we will test if the depressive-like phenotype is expressed. To do so, we will test affective-related, cognitive, and social behaviours of F2 as well as molecular mechanisms involved, by proceeding the same as we will proceed in study 1. Therefore, the same order and type of behavioural tests, as well as mRNA expression analysis and DNA methylation analysis will be conducted.

The chronology of this study is summarised in fig5:

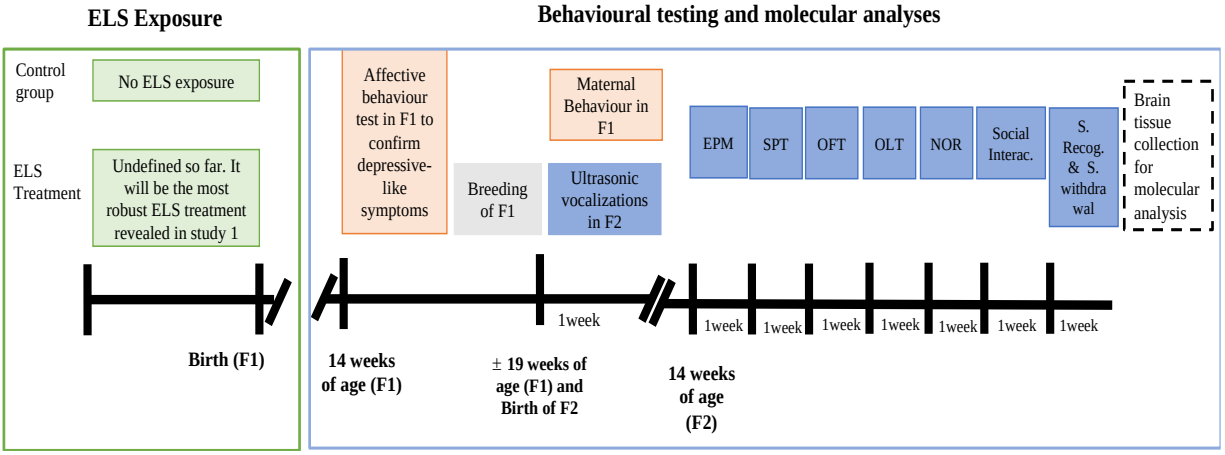

Fig5. General design of study 5. The symbol ± means approximately. Successful breeding of F1 may take longer. It is possible that depressed-like females will not get pregnant in the first attempt of breeding.

F1 and F2 outcomes will be analysed separately.

F1 analysis:
To analyse affective-related behaviours and maternal care of F1, two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

The primary outcome parameter to confirm the depressive-like phenotype in F1 will be the responses that they will display in EPM, SPT and OFT.

The primary outcome parameter to test altered maternal care in F1 females will be the responses of care towards the pups expressed by the dam in the nest. Altered maternal care in humans is a clinical feature of postpartum depression (a type of depression); therefore, we will test whether the depressive-like phenotype in F1 females alters the maternal care.

F2 analysis:
To analyse altered social communication, depressive-like phenotype, gene expression, and epigenetic marks of F2, four conditions, one control and three experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats born from $SERT^{+/-}$ F1 males mated with control $SERT^{+/+}$ F1 females.

Experimental condition 1: SERT$^{+/+}$ rats born from early-life stressed SERT$^{+/+}$ F1 males mated with control SERT$^{+/+}$ F1 females.

Experimental condition 2: SERT$^{+/+}$ rats born from early-life stressed SERT$^{+/+}$ F1 females mated with control SERT$^{+/+}$ F1 males.

Experimental condition 3: SERT$^{+/+}$ and SERT$^{+/-}$ rats born from early-life stressed SERT$^{+/-}$ F1 males mated with control SERT$^{+/+}$ F1 females.

Experimental condition 4: SERT$^{+/+}$ and SERT$^{+/-}$ rats born from early-life stressed SERT$^{+/-}$ F1 females mated with control SERT$^{+/+}$ F1 males.

The primary outcome parameter to test altered social communication in F2 pups induced by the F1 depressive-like phenotype will be the ultrasonic vocalizations emitted by the pups. The selection of this test is based on the natural ability of pups to transmit affective-related states to their mother through ultrasonic vocalizations. Reduced ultrasonic vocalizations of F2 will be an indicator of dysfunctional social communication very early in life as a result of being born from depressed-like parents.

The primary outcome parameters to test the F2 depressive-like phenotype induced by F1 parental-like depression will be the responses displayed by F2 rats in the behavioural tests indicated above (also shown in the blue boxes of fig5).

The primary outcome parameters to test changes in gene expression and epigenetic markers of F2 induced by F1 parental-like depression will be the level of mRNA expressed of selected genes and the level of DNA methylation at the promotor regions of selected genes in brain regions that are known to be involved in depression.

The selection of these behavioural tests and molecular analyses is based on evidence of offspring neurobiological and behavioural outcomes associated with stress/depression in the mother [14-16] and the father [17,18]. It is suggested that not only maternal stress can induce epigenetically driven effects on the offspring, but also paternal stress can induce long-lasting changes in germ cells, thus potentially inducing changes across generations [17].

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

Study 1: the depressive-like phenotype by *cumulative* ELS
SERT$^{+/+}$ and SERT$^{+/-}$ rats will be exposed to *cumulative* ELS. The procedures to induce (maternal) pregestational ELS, (maternal) prenatal ELS, and postnatal ELS are described below:

1) *pregestational ELS* refers to stressful events experienced by females before pregnancy to interfere with the development of the offspring. Pregestational stress will be applied to females for at least 21 days before pregnancy, induced by chronic unpredictable stress. We will follow the same protocol as [19,20]. Based on this protocol, females will be housed individually and subjected to 1–2 stressors per day for 3 weeks. Stressors will include restraint under bright light (1000 lux) for 1 h, overcrowding overnight (4 females per Macrolon type III cage), overnight exposure to damp bedding, 12h of food restriction, 5 minutes of forced swimming, and cage rotation for 12h. In accordance with the *Nationaal Comité advies dierproevenbelid* advice to prevent withdrawal of food for more than 24 hours in animals used for neurocognitive research [21], 12 hours of food restriction will be the maximum time used in this procedure and the female will be exposed to environmental enrichment; in addition, food restriction will not be applied in two consecutive days. Cage rotation will consist of changing the home cage location from one place to another from morning to afternoon, within the same experimental room. We select chronic unpredictable stress due to its previous validation to induce sustained changes in the stress response and depression-like responses in rodents. This procedure has been effective to induce neural changes and behavioural impairments in the offspring when it is applied to females before pregnancy. It was shown that pregestational stress caused lower viability of pregnancy, so about 1/3 of females did not became pregnant [22]. However, we will do some rebreeding attempts (max 3 attempts) to cover that.

2) *prenatal ELS* refers to stressful events experienced by females during their pregnancy to interfere with the development of the offspring. We will follow the same protocol as van den Hove et al. 2006 [23]. Females will

be exposed to stress during the last week of pregnancy (14-21days), subjected to 3 sessions of 45-min restraint stress each day while being exposed to bright light. Dams will be put inside a plastic tube in which they can move their paws but cannot turn around. Each 45-min stress session will be as unpredictable as possible. We select restraint stress due to its efficacy to induce changes in the stress response and increase the likelihood of expressing depresssive-like behaviours. This procedure has been effective in inducing neural changes as well as affective-related and social impairments in the offspring when it is applied to females in the last week of pregnancy. Based on the van den Hove et al procedure, prenatal stress had no effect on gestational length, litter size, or pre-weaning mortality [23].

3) *postnatal ELS* refers to stressful events experienced during the first weeks of life (in rodents). In this research project, $SERT^{+/+}$ and $SERT^{+/-}$ rats will be exposed during the first two weeks of life (PND 2-15) to unpredictable maternal separation and disrupted maternal care induced by maternal stress. We will follow the same protocol as [4]. Unpredictable maternal separation in combination with maternal stress produces more persistent behavioural effects in the offspring. As a consequence of disrupted maternal care, the offspring is at higher risk of developing affective-related impairments later in life.

To induce unpredictable maternal separation, pups will be transferred as a whole litter into a new room for 3h/per day, starting at unpredictable time points each day. The whole litter will be placed in preheated Makrolon type II cages to prevent hypothermia (postnatal days 1-8: 32±1 °C; postnatal days 9-15: 28±1 °C). To disrupt maternal care behaviours, mothers will be exposed to maternal stress. Maternal stress will consist of either 20-min restraint stress or 5-minute forced swimming in cold water. They will be applied unpredictably and randomly during the separation of the litter. For restraint stress, dams will be put inside a plastic tube in which they can move their paws but cannot turn around. For the forced swimming, females will be placed into a cylindrical Plexiglas tank filled up with water at 18 °C.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. From 14 weeks of life, $SERT^{+/+}$ and $SERT^{+/-}$ rats (2 males and 2 females maximum per litter) will be behaviourally tested. General procedure for each behavioural test is indicated below:

1) EPM: The rat will be taken from the home cage and placed in the centre of the maze facing an open arm. The rat will be allowed to freely explore the maze for 5 min. Afterwards, the animal will be returned to the home cage.
2) SPT: Within his/her home cage, the rat will be exposed to one bottle of water and one bottle containing a sucrose solution for 24h on alternating days. On the other days two bottles of water will be presented. With each sucrose day, the sucrose concentration will be increased. Sucrose bottle locations on the cage will be alternated on sucrose days to prevent spatial bias. This test will take place for one week in total.
3) OFT: The rat will be taken from the home cage and placed in the centre of the open arena to freely explore the open field for 10 minutes. Afterwards, the animal will be returned to the home cage.
4) OLT: The rat will be taken from the home cage and placed into an open arena with two identical objects placed in two opposite corners. Free exploration will be allowed for 3 min (trial 1). After that, both the rat and the objects will be removed for 1 hr (to test short-term memory), after of which the next trial will start (trial 2). In this trial, the animal will be exposed to the same two objects as trial 1 for another 3 min; however, this time one of the objects will be place in a novel location. After the trial 2 is over, the animal will be returned to the home cage.
5) NOR: The procedure will be the same as for OLT. In this case, trial 1 will consist of exposure to two identical objects whereas trial 2 will consist of exposure to one familiar and one novel object placed in the same location during both trials.
6) Social interaction test: After the habituation to the arena, two rats that are unfamiliar to each other will be placed simultaneously in an open arena for 10 minutes. Each couple of animals will be matched by sex, SERT genotype, and ELS treatment. Afterwards, both animals will be returned to the home cage.
7) Social recognition test. After 10-minute habituation, the experimental rat will be allowed to explore an unfamiliar younger stimulus rat (stranger 1) that will be placed under a plastic grid in the left or right chamber of a three-chamber box, while the other chamber will contain an empty grid. After 10-minute exploration, a second unfamiliar younger stimulus rat (stranger 2) will be placed in the empty grid, and 10-minute exploration will be allowed. Afterwards, the animal will be returned to the home cage.

After all behavioural tests have been taken place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Study 2: focus on social functioning in the juvenile period
$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. At ±4-5 week of age, animals will be assessed for social play behaviour in the social play-fighting test. For this test, animals will be tested in couples. Each couple will be considered as an experimental unit and will consist of two unfamiliar mates matched by sex, SERT genotype, and ELS treatment. After the habituation to the testing cage (5 minutes), animals will be tested for 15 minutes. Boxing/wrestling, pouncing, pinning, chasing and social grooming will be scored.

After 90-120 minutes the social play-fighting test is over, the rat will be taken to a different room and will be anesthetised. Then, the rat will be sacrificed by perfusion and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Study 3: focus on social functioning in adulthood
$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. As of 14 weeks of age, sexual behaviour of males and females will be tested once a week for 6-10 consecutive weeks. To test sexual behaviour in males, we will place the rat with a stimulus female in a testing cage for 30 minutes. To test sexual behaviour in females, we will place the rat with a stimulus male in a testing cage for 30 minutes. Table 1 below indicates the sexual responses that will be scored in males and females. Immediately after the test, rats will be returned to their home cage.

After the last sexual test has been taken place, males will be housed individually with a companion female in a new home cage to elicit territorial behaviour. Likewise, females will be housed individually in a new home cage to elicit territorial behaviour. Water and food will be provided *ad libitum*. For home cage enrichment, wooden gnawing sticks, a polycarbonate small box for resting/sleeping and autoclaved bedding material will be provided. One week later, aggressive behaviour will be tested. For this assessment, we will use the resident-intruder test in males, and the female-intruder test in females.

The resident-intruder test will consist of the introduction of an unfamiliar, intruder male into the home cage of the resident male. The female-intruder test will consist of the introduction of an unfamiliar, intruder female into the home cage of the resident female. In both cases, the resident will correspond to the experimental animal, while the intruder will correspond to a same-sex, smaller stimulus rat that is unknown for the experimental animal. The aggression test will take place for 30 minutes and the behaviour of the resident will be recorded. Table 1 below indicates the aggressive responses to be scored in males and females. Immediately after the aggression test is over, rats will be placed in their home cage. From 90 to 120 minutes after the aggression test has been taken place, the rat will be taken to a different room and will be anesthetised. Then, the rat will be sacrificed by perfusion and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Table 1. Readouts of sexual behaviour and aggressive behaviour

Sexual behaviour	**Aggression**
- Copulatory behaviour in males by scoring mounts, intromissions, and ejaculations - Receptive behaviour in females by scoring lordosis - Proceptive behaviour in females by scoring darts and hops and time spent with the male	- Attacks, lateral threat, upright posture, clinch attack, keep down and chase in males during the resident-intruder test. - Same parameters in females during the female-intruder test.

As aggressive encounters induce a stress response in animals, we will evaluate the stress response induced by the resident-intruder test in males and the female-intruder test in females. We will collect three blood samples

from the animals' tail to assess the concentration level of corticosterone (the main stress hormone in rodents) in systemic circulation. Sampling will be made by making a small cut in the tail and collecting the blood from the dorsal tail vein. The first sample will be collected before the test to establish the baseline level of the hormone. The second sample will be collected immediately after the aggression test by removing the crust from the incision and collecting the blood. The third sample will be collected 30 minutes later by removing the crust (or making a new, small incision if needed) and collecting the blood. Each sampling will be maximum 300 uL, so the total amount of blood sampling will be < 1ml/kg. This procedure is considered as stress-free procedure and has been proven to be reliable to test corticosterone levels in systemic circulation in rodents [24].

Additional animal procedures in this study:
To prevent pregnancy in experimental females when the sexual behaviour is tested with the stimulus male, double tubal ligation surgery will be performed. This surgery will be also performed in stimulus females used to test sexual behaviour of experimental males. Likewise, same surgery will be performed in companion females that will be caged with experimental males to conduct the resident-intruder test. In all three cases, the double tubal ligation surgery will be performed at least one week before the female is in contact with the male.

In preparation for the surgery, the rat will be anesthetised. To minimise discomfort directly linked to the surgery, analgesics will be subcutaneously injected at the start of the surgical procedure. The female will receive analgesics 24 hrs and 48 hrs hours after surgery. During the 7- 14-day recovery period, the weight and the well-being of the animals will be checked daily (on weekdays). Stimulus females used to test sexual behaviour of experimental males and companion females caged with experimental males for the resident-intruder test will be primed by subcutaneous injection of oestradiol to be behaviourally receptive while interacting with males.

Study 4: unravelling molecular mechanisms of social functioning
$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. The animals will be left undisturbed (except for cage cleaning) until ±24 week of age in which they will be sacrificed by rapid decapitation after CO_2 asphyxiation and brain tissue will be immediately collected and stored until the molecular analyses indicated in the previous section are performed.

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
$SERT^{+/+}$ and $SERT^{+/-}$ F1 rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1. ($SERT^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, $SERT^{+/+}$ rats will be used).

After ELS exposure, F1 rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype and ELS treatment. From 14 weeks of age (similar to study 1), we will test F1 affective-related behaviours by using the same tests used in study 1 (i.e., EPM, SPT and OFT) and following the same procedure. After the last behavioural test, F1 animals will be mated. To do so, depressed-like males and depressed-like females will be mated with wildtype, control females and males, respectively, to produce F2.

F1 pregnant females will be left undisturbed during pregnancy (except for cage cleaning). After delivery of F2, we will measure social communication of F2 pups on postnatal day 6 and maternal care of F1 females during the first postnatal week of F2.

Social communication of F2 pups will be measured by ultrasonic vocalizations produced by the pup in response to separation from the dam and littermates in a 5-minute test. The pup will be individually transported from the nest to a testing room in which no other animals will be present. The pup will be place in a Makrolon type 2 cage filled with Aspen wood chip that will be under the ultrasonic microphone.

Maternal care of F1 females will be tested by scoring care behaviours displayed by the dam towards the pups in the nest, three times a day -30 minutes each- during the first postnatal week of F2. Licking/grooming,

arched-back nursing and contact with pups will be scored in the home cage to minimise any disruption to the dams (F1) or the offspring (F2).

F2 rats will be weaned at postnatal day 21 and socially housed with mates matched by experimental condition (see previous section about description of general design for further explanation of control and experimental conditions). From 14 weeks of age (similar to study 1), we will test whether the depressive-like phenotype is expressed in F2. Therefore, we will test F2 affective-related, cognitive, and social behaviours and molecular mechanisms involved by proceeding the same as we will proceed in study 1. After all behavioural tests have been taking place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

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

To estimate the sample size of each study, we used the Gpower 3.1 statistical software. The input parameters to calculate the sample size of each study for ANOVA (fixed effects, special, main effects, and interactions) statistical tests depended on three aspects: 1) the number of groups per study; 2) assumptions of power and alpha for statistical significance and, 3) the effect sizes reported in previous studies.

Aspect one: The number of groups required per study:

- Study 1 = 16 groups (4 treatments, 2 SERT genotypes, 2 sexes)
- Study 2 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 3 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 4 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)

As in study 5 two generation of rats will be tested (i.e., F1 and F2), the estimation of animals for this study is as follow:

- F1 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes).
- F2 = 16 groups (4 parental conditions, 2 SERT genotypes, 2 sexes). See previous section about description of general design, study 5, for further explanation of control and experimental conditions of F2.

Aspect two: We based our calculations on the assumption to obtain a significant group effect with a power of 80% and alpha value of 0.05.

Aspect three: For behavioural studies 1, 2, 3, and 5, we used the effect sizes from behavioural results reported by 5.1 lid2e, 5.1 lid2h [4]. Even though we do not know the behavioural outcomes of our proposed studies, especially of animals exposed to the cumulative ELS, we consider the effect sizes from findings reported by 5.1 lid2e, 5.1 lid2h as appropriate for our estimations as they also studied the interaction of ELS and the SERT genotype to test animals' behaviour and used several of the same tests we have proposed here. For study 4, we used the effect size based on the findings reported by 5.1 lid2e, 5.1 lid2h [25]. Even though we do not know the molecular outcomes of animals exposed to ELS in our proposed study, we consider the effect size from findings reported by 5.1 lid2e, 5.1 lid2h appropriate for our estimations as they also studied the exposure to ELS as one factor influencing the gene expression and epigenetic regulation of some of the same brain regions we have proposed to analyse here.

Other considerations to calculate the number of animals:

1- Animals to be tested in all studies are born from mothers exposed to stress, therefore, we also estimated the number of females to be exposed to stress. This calculation is described in section B of this document, number of animals.
2- Animals to be used in studies 2 to 4 will be born from the same mothers. Therefore, we will assign 1 male and 1 female per litter to each study. On one hand, this will maximise the use of the offspring; on the other, it will control litter effects for molecular analysis involved in studies 2 to 4. Even though we do not know the behavioural outcomes of studies 2 and 3, the brains for study 4 will be collected to optimise animal use (use of the same litters from studies 2 and 3).

Minimising the number of animals in studies 2 to 5 might be possible based on outcomes of study 1. If results from this study indicate that $SERT^{+/-}$ rats are not more vulnerable than $SERT^{+/+}$ animals, we will not use this knockout manipulation for studies 2 to 5; therefore, the number of animals needed for those studies might be reduced.

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
A2	Rattus norvegicus	Animals bred in our animal facility	Adult females to expose to stress and breed. Newborn, juvenile, and adult offspring to test behaviour and for molecular analysis	3129	Both	Yes	Wistar

Provide justifications for these choices

Species	Affective-related, cognitive and social behaviours in the rat resemble functional similarities of the same behaviours in humans. Similar to humans, rats are also highly sensitive to stress exposure very early in development. Changes in rat behaviour following *cumulative* ELS can resemble human behavioural changes after a long history of ELS. In addition, the brain circuit for the expression of affective-related, cognitive and social behaviours is highly conserved across mammals. Therefore, brain circuit functionality in the rat resembles brain circuit functionality in humans.
Origin	**Study 1:** F0 = $SERT^{+/-}$ males mated with $SERT^{+/+}$ females. $SERT^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = $SERT^{+/+}$ and $SERT^{+/-}$ rats born from F0 **Studies 2 to 4:** F0 = $SERT^{+/-}$ males mated with $SERT^{+/+}$ females. $SERT^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups. This generation will be the parents of rats to be used in studies 2 to 4 F1 = $SERT^{+/+}$ and $SERT^{+/-}$ rats born from F0 **Study 5:** Origin of animals for control condition: F0 = $SERT^{+/-}$ males mated with control $SERT^{+/+}$ females. $SERT^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = $SERT^{+/+}$ and $SERT^{+/-}$ rats born from F0, and mated with control $SERT^{+/+}$ rats to created F2 F2 = $SERT^{+/+}$ and $SERT^{+/-}$ rats born from F1 Origin of animals for experimental conditions 1 and 2: F0 = $SERT^{+/+}$ males mated with stressed $SERT^{+/+}$ females F1 = stressed $SERT^{+/+}$ rats born from F0, and mated with control $SERT^{+/+}$ rats to create F2 F2 = $SERT^{+/+}$ rats born from stressed $SERT^{+/+}$ F1 rats Origin of animals for experimental conditions 3 and 4: F0 = $SERT^{+/-}$ males mated with stressed $SERT^{+/+}$ females F1 = stressed $SERT^{+/-}$ rats born from F0, and mated with control $SERT^{+/+}$ rats to create F2 F2 = $SERT^{+/+}$ rats and $SERT^{+/-}$ rats born from stressed $SERT^{+/-}$ F1 rats

Life stages	**Study 1** *Adult females:* for stress exposure and breeding at 3 months of age *Adult offspring:* for testing the depressive-like phenotype and molecular analysis of brain tissue **Studies 2 to 4** *Adult females:* for stress exposure and breeding at 3 months of age *Juvenile offspring to study 2:* for social play testing and molecular analysis of brain tissue *Adult offspring to study 3:* for sexual behaviour and aggression testing and molecular analysis of brain tissue *Adult offspring to study 4:* for molecular analysis of brain tissue at the same age of rats used in study 3 to make outcomes of molecular analysis comparable **Study 5** *Adult females* (F0): for stress exposure and breeding at 3 months of age *Adult offspring* (F1): for testing the depressive-like phenotype and breeding *Newborns and adult offspring* (F2): for testing transgenerational behavioural effects of parental depressive-like phenotype and molecular analysis of brain tissue

Number

Study 1:
We will need n=14 animals per group; and we will have 16 groups (4 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 14 x 16 = 224. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed 224 / 4 = 56. We estimated 10 pups delivered by each F0 female, so 56 x 10 = 560. If we will use 2 male and 2 female pups per litter to get the total sample of 224 rats, 6 pups per litter will not be used (56 X 6 = 336). See calculations per treatment as follows:

F0 females exposed to ELS N=56				F1 rats used (2 males + 2 females per litter) N= 224				F1 rats exposed to ELS non-used for study 1 (6 pups per litter) N= 336			
Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3
14	14	14	14	56	56	56	56	84	84	84	84

Abbreviations: ELS-T1 = Postnatal stressors; ELS-T2 = Prenatal and postnatal stressors; ELS-T3 = Pregestational, prenatal and postnatal stressors.

Exposure to stress in F0 females can interfere with normal body weight, affect their welfare, reduce the rate of fertilization, or alter pregnancy maintenance, especially in females exposed to ELS-T3. Therefore, we estimated extra F0 females to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations according to the 1/3 of non-pregnancy rate reported by Gemmel et al [22]; hence we estimated 30% extra for ELS-T3. We estimated 15% extra for ELS-T2 and ELS-T1 each based on possible (although unlikely) reduced gestational length, litter size, or pre-weaning mortality [23]. In addition, extra F1 pups born from extra F0 females were also estimated to be used because stressed mothers might deliver fewer than 10 pups per litter, or animals born from stressed mothers might not survive due to impaired maternal care (especially ELS-T3. See calculations of all extra rats per treatment as follows:

Extra F0 females for possible dropouts N=11				Extra F1 rats for possible dropouts (2 males + 2 females per litter) N= 44				Extra F1 rats exposed to ELS non-used for study 1 (6 pups per litter) N= 66			
Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3
0	3	3	5	0	12	12	20	0	18	18	30

Studies 2 to 4:
For study 2, we will need n=16 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 16 x 8 = 128.

For study 3, we will need n=16 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 16 x 8 = 128.

For study 4, we will need n=9 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 9 x 8 = 72.

Animals needed for studies 2 to 4 will come from the same litter and be born from dams (F0) exposed to stress. Therefore, the total number of F0 females needed is = 64. We estimated 10 pups delivered by each F0 female, so 64 x 10 = 640. If we will use 128 rats to the study 2, 128 rats to the study 3, and 72 rats to the study 4 (to get a total of 328 rats), the total of non-used animals will be =312. See calculations per treatment as follows:

	F0 females exposed to ELS N= 64		F1 rats used N=328		F1 rats exposed to ELS non-used for studies 2 to 4 N=312	
	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
Study 2	32	32	64	64	156	156
Study 3			64	64		
Study 4			36	36		

As in study 3 surgery for tubal ligation will be performed in females, we also estimated extra females to be used for possible dropouts related to this procedure. Therefore, if we will have 4 groups of females (2 treatments, 2 SERT genotypes), we estimated one extra female per group, therefore, n of dropouts=4

We also estimated extra F0 females and extra F1 pups to be used for possible dropouts for same reasons indicated above. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations assuming that the most robust ELS will be the ELS-T3; hence we estimated 30% extra. See calculations of all extra rats as follows:

	Extra F0 females for possible dropouts N= 10		Extra F1 rats for possible dropouts N=52		Extra F1 rats exposed to ELS non-used for studies 2 to 4 N=48	
	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
Study 2	0	10	0	20	0	48
Study 3			0	20		
Study 4			0	12		

The estimated number of companion females to test males' sexual behaviour is = 64
The estimated number of companion females to be housed with males for aggression test is = 64

Study 5:
Estimated number of F1 rats
We will need n=20 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 20 x 8 = 160. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed is 160 / 4 = 40. We estimated 10 pups delivered by each F0 female, so 40 x 10 = 400. If we will use 2 male and 2 female pups per litter to get the total sample of 160 rats, 6 pups per litter will not be used (40 X 6 = 240). See calculations as follows:

F0 females exposed to ELS N=40		F1 rats for behavioural testing and breeding N=70		F1 rats for behavioural testing only N=90		F1 rats exposed to ELS non-used for study 5 N=240	
Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
20	20	14	56	66	24	120	120

We also estimated extra F0 females and extra F1 pups to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations assuming that the most robust ELS will be the ELS-T3; hence we estimated 30% extra. See calculations of all extra rats as follows:

Extra F0 females for possible dropouts N=6		Extra F1 rats for possible dropouts (2 males + 2 females per litter) N=24		Extra F1 rats exposed to ELS non-used for study 5 (6 pups per litter) N= 36	
Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
0	6	0	24	0	36

Estimated number of F2 rats

We will need n= 14 rats per group; and we will have 16 groups (4 parental conditions, 2 SERT genotypes, 2 sexes); therefore, total sample size is 14 x 16 = 224. The parents of these animals will be F1 rats used for testing and breeding, indicated in the table above (N=70). We estimated 10 pups delivered by each F1 parent, so 70 x 10 = 700. If we will use 56 control animals and 168 experimental animals to get the total sample of 224 rats, n=476 will not be used. See all calculations below:

F2 rats used for behavioural testing and brain collection N=224		F2 rats born from F1 non-used for study 5 N= 476	
Controls	Experimental groups	Controls	Experimental groups
56	168	84	392

We also estimated extra F2 animals born from extra F1 rats because animals born from F1 depressed-like mothers might not survive due to impaired maternal care, or litters from $SERT^{+/-}$ parents might be smaller. See calculations as follows:

Extra F2 rats born from extra F1 animals N=96		Extra F2 born from extra F1 non-used for study 5 N= 144	
Controls	Experimental groups	Controls	Experimental groups
0	96	0	144

Sex	Depression in humans is expressed in both sexes, therefore we will conduct this research in male and female rats.
Genetic alterations	$SERT^{+/-}$ rats that express lifetime low expression of the serotonin transporter (SERT) will resemble low SERT expression in human carriers of the short allele in the SERT gene.
Strain	Wistar

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.

- In study 3, one or two tail incisions (in case taking off the crust from the first incision does not work) will be made to collect the blood as described in section A of this form. Pain relieving will not be used for this procedure because the severity is mild and, if properly executed, it is not expected to cause any detectable adverse effect.
- Studies 1 and 4 involve brain tissue collection after decapitation to perform molecular analysis. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible. Anaesthesia is not recommended before decapitation because it can alter the analysis of gene expression.

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

- Studies 2 and 3 involve brain tissue collection after perfusion to perform analysis of c-Fos expression. Preparation for perfusion will involve an intraperitoneal overdose of pentobarbital for deep anaesthesia and non-recovery. The dose will be adjusted for each animal's weight.
- For double tubal ligation to females in study 3, each female will be under anaesthesia. To minimise discomfort directly linked to the surgery, analgesics will be subcutaneously injected. The female will receive analgesics 24 hrs and 48 hrs hours after surgery. During the 14-day recovery period, the weight and the well-being of the animals will be daily checked.

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

- Chronic unpredictable stress applied to females before conception may reduce their body weight and/or interfere with fertilization.
- Restraint stress in pregnant females may affect their body weight, the maintenance of pregnancy, the number of offspring alive after delivery and/or litter size at birth.
- Unpredictable maternal separation and disrupted maternal care may potentially increase pups' mortality and/or may reduce the body weight of both mothers and the litter.
- Isolation of females 1 week prior to female-resident intruder test may induce stress (study 3)

Explain why these effects may emerge.

- Prolonged exposure to stress dysregulates the release of glucocorticoids. This interferes with the normal activity of glucose, thus affecting the metabolism of animals. In addition, high levels of glucocorticoids induced by stress can interfere with normal functioning of sexual hormones associated with embryo implantation.
- Exposure to high levels of stress during pregnancy increases the levels of glucocorticoids into systemic circulation that can interfere with the functioning of the placenta or foetal development. This situation can alter the maintenance of gestation, normal foetal growth, or number of pups alive after delivery.
- Separation from mothers or disrupted maternal care may reduce the amount or quality of mothers-litter nurturing, therefore, these procedures may reduce the amount or quality of nesting, lactation, or mothers' licking behaviour towards their litters, all of which are necessary for pups' development.
- Social isolation is stressful for social animals.

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

- We will do 3 attempts of rebreeding (maximum) as soon as the pregestational stress procedure is over. Pregestational stress procedure can finish 0-2 weeks before the gestational period begins without interfering with the experimental outcome [26].
- We will provide sufficient food and water ad libitum to maintain body weight as normal as possible.
- We will provide enough bed material, gnawing sticks and group housing for environmental enrichment that helps to cope with stress.
- We will provide food and water ad libitum, enough bed material and gnawing sticks for environmental enrichment for isolated females in study 3.
- We will monitor that animals are not exposed to sources of stress other than stressors used for the experiments. This means we will prevent a noisy environment, inadequate room temperature, light, or ventilation, inadequate handling, etc.

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.

If animals show clear signs of the sickness behaviour and/or display grimace signs of pain (arched back, rough coat, general malaisse) or loose more than 15% of their body weight in 48 hours, they will be excluded from the experiment and terminated. If they show tumors, they will be terminated. We will not interfere with the pups until they are weaned (that is, outside the maternal separation, we will leave the nest with the mom).

Indicate the likely incidence.

Very low. Although stress procedures are chronic, our previous experience as well as research conducted elsewhere have shown that the level of discomfort induced by selected stress procedures do not significantly interfere with feeding, water consumption, motor activity, or cleaning behaviours. Based on van den Hove et al [23] procedure, prenatal stress had no effect on gestational length, litter size, or pre-weaning mortality, so restraint stress effects on pregnant rats are very unlikely. As far as we know, nobody has performed studies combining pregestational, prenatal and postnatal ELS, therefore, we are not sure about the incidence of negative effects. With drug treatments (maternal fluoxetine use) we used before we saw viability index of 72-73%. We expect these stressors to have a higher viability index as we saw that only one ELS had no significant effects [Chapter 5 in ref 4]. If 28% would die, we still have enough animals left in the litter. We will monitor the welfare of the females and offspring to properly record it and take actions to implement humane endpoints if applicable.

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).

Of a total of estimated animals to be used, it is expected that 22% of animals are exposed to cumulative mild discomfort and 78% are exposed to cumulative moderate discomfort.

	All studies		Study 1		Study 2		Study 3		Study 3		Study 4		Study 5		Study 5		Companion females housed with males + stimulus females to test males' sexual behavior in study 3	All studies		Study 5	
	F0		F1		F1		F1 males		F1 females		F1		F1		F2			Non-used F1 rats		Non-used F2 rats	
	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	EXP		CTR	ELS	CTR	EXP
Arrival	2	2																			
Handling	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2				
Stress exposure		3		3		3		3		3		3		3					3		
Cognitive tests			2	2											2	2					
Affective tests			2	2									2	2	2	2					
Social tests			2	2	2	2	3	3	3	3					2	2					
Home accommodation to induce territorial behaviour							2	2	3	3											
Repeated blood sampling							3	3	3	3											
Tubal ligation surgery									3	3							3				
Primed s.c. oestradiol treatment (max 1x every two weeks)																	2				
Euthanasia	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
Cumulative discomfort (SV)	SV2	SV 3	SV3	SV3	SV2	SV3	SV3	SV3	SV3	SV3	SV2	SV3	SV2	SV3	SV3	SV3	SV3	SV2	SV3	SV2	SV3
% of animals to have expected cumulative discomfort	2.1	3.9	1.8	6.8	2.0	2.7	1	1.3	1.1	1.4	1.1	1.5	2.6	3.3	1.8	8.5	4.1	11.5	21.7	2.7	17.1

***** *EU directive scale: SV1=terminal, SV2=mild, SV3=moderate, SV4=severe*

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	The measurement of the depressive-like phenotype in the rat model implies the assessment of complex patterns of behaviours to resemble the human condition. *In vivo* studies conducted with rats allow to investigate a wide range of behaviours that are functionally similar to those observed in humans, as well as the underlying mechanisms in the brain. Additionally, the functioning of brain regions involved in regulation of affective-related, cognitive, and social behaviours in humans is highly conserved in rats and cannot be found in lower organisms; therefore, the use of these types of live animal experiments is still irreplaceable. Moreover, *in vitro* studies on isolated tissue would not be able to give a sufficient overview of the effect of *cumulative* ELS in the brain and behaviour in the offspring. In short, it is not feasible to replace the use of live animals in this research project.
Reduction	To assess the effects of cumulative ELS and SERT gene interaction, intact and freely behaving rats will have to be used. We have developed in-depth expertise in the proposed animal experimental paradigms; in addition, we have selected validated, optimised, and refined protocols that will reduce the number of animals needed to obtain significant results. The number of animals needed for these studies will be carefully considered based on prior studies and expected variance in the dependent variables. Furthermore, we consider carefully which models are needed for our studies. First, the go/no go moments are described in the section 3.4.1 of the proposal form. If the depressive-like behaviours are not expressed in animals due to any of the ELS treatments used in study 1, following studies will no longer be relevant so no more animals will be used. If $SERT^{+/-}$ rats do not express higher vulnerability to depressive-like behaviours than $SERT^{+/+}$ rats, they will no longer be used in studies 2 to 5. Hence, only $SERT^{+/+}$ rats will be included and the number of groups and animals in studies 2 to 5 would be reduced. Second, estimated number of animals per group per study is based on statistical methods aimed at minimising the number of animals. Third, the offspring of studies 2 to 4 will come from the same parents, thus maximising the use of the offspring.
Refinement	All procedures are used regularly in our laboratory and have been previously refined to minimise the potential discomfort. The responsible researchers have ample expertise with these procedures and with training other researchers. The animal housing facilities are well-equipped to house rodents. The responsible researchers and the staff from the animal facilities are properly trained to handle the animals; assess the health and welfare of the animals; administer anaesthesia and minimise pain and suffering. All surgical procedures will be performed under anaesthesia, with proper post-operative care. Animals will be humanely killed at defined end points according to national ethical rules. Stress procedures will produce discomfort in animals. However, ad libitum food and water (unless stated otherwise) and environmental enrichment through social housing, gnawing sticks, and sufficient nesting material in the home cage in all studies will be ensured to increase strategies to cope with stress.

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.

In study 5, non-stressed F1 rats can be re-used for educational purposes as discomfort in them is classified as mild. However, if they are declared unfit for further use by the designated veterinarian (Art 14), killing will be done according to EU guidelines. Animals will only be re-used if they, at the end of the study, are suitable for other experiments covered by an existing CCD license or studies that are below threshold; if not, we will sacrifice them.

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

For studies 1 and 4, killing by decapitation is required for brain collection. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible.

For studies 2 and 3, killing by perfusion is required for brain collection. Intraperitoneal overdose of pentobarbital for deep anaesthesia and non-recovery will be used.

For study 5, F1 animals will be sacrificed after completing the behavioural tests and/or breeding as they have fulfilled the scientific purpose and their cumulative discomfort is classified as moderate. F2 animals of study 5 will be killed by decapitation for brain collection. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible.

Companion females will be sacrificed after completing study 3 as they have fulfilled the scientific purpose and cumulative discomfort is classified as moderate.

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 will be used in F0 rats and non-used animals of all studies. It will be used also in companion females and F1 rats of study 5. The animal will be transported to a gas chamber in which CO_2 flow will be allowed until pressumed death is confirmed.

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

Decapitation will be used in animals of studies 1, 4 and 5 to collect brain tissue and analyse gene expression level and epigenetic markers. Decapitation is selected because fresh frozen tissue samples are preferred to analyse gene expression level by using the quantitative polymerase chain reaction (RT-qPCR) technique. Prior to decapitation, the rat will be taken to a different room and will be exposed to CO_2 asphyxiation produced from dry ice until sedation. Immediately after, the animal will be decapitated by using a guillotine.

Perfusion will be used in animals of studies 2 and 3 to collect brain tissue and analyse the c-Fos expression level. Perfusion is preferred to maximise good quality of brain slices to perform immunohistochemistry staining for c-Fos quantification. Prior to perfusion, the rat will be anaesthetised with an overdose of pentobarbital. Immediately after, a transcardiac perfusion with saline followed by 4% paraformaldehyde will be performed to clear blood and preserve the brain tissue.

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

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Format DEC-advies

A. Algemene gegevens over de procedure

1. Aanvraagnummer 5.1 lid2h **/ AVD**5.1 lid2h **202317174**

2. Titel van het project: **Transgenerational susceptibility to depression due to early life stress**

3. Titel van de NTS: **Transgenerationele gevoeligheid voor depressie door stress in het vroege leven**

4. Type aanvraag:
   - ☐ **nieuwe aanvraag projectvergunning**

5. Contactgegevens DEC:
   - naam DEC: 5.1 lid2h
   - telefoonnummer contactpersoon: 5.1 lid2e
   - e-mailadres contactpersoon: 5.1 lid2h

6. Adviestraject (data dd-mm-jjjj):
   - ☐ ontvangen door DEC: **04-07-2023**
   - ☐ aanvraag compleet: **04-07-2023**
   - ☐ in vergadering besproken: **06-07-2023 en 12-10-2023**
   - ☐ anderszins behandeld: **overleg met aanvrager in 'grote' DEC 17-08-2023, zie A8**
   - ☐ termijnonderbreking(en) van / tot **06-07-2023 – 05-10-2023**
   - ☐ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen **21-08-2023**
   - ☐ aanpassing aanvraag **05-10-2023**
   - ☐ advies aan CCD **02-11-2023**

Deze aanvraag wordt door de 5.1 lid2h **gezien als 'complex': de** 5.1 lid2h **was van mening dat naast het mogelijke cumulatieve ernstige ongerief van de individuele dieren, het nut en de noodzaak van de voorgestelde proeven niet duidelijk verwoord waren. Daarom heeft de** 5.1 lid2h **op 6 juli de aanvrager uitgenodigd voor een open gesprek tijdens de DEC vergadering van 17 augustus 2023. De** 5.1 lid2h **heeft eerder op 9 juli een lijst met vragen en opmerkingen ter voorbereiding voor een grote DEC naar de aanvrager gestuurd. In de vergadering van 17 augustus heeft er een open gesprek plaats gevonden tussen de aanvrager en de** 5.1 lid2h **Daarin heeft de aanvrager aangegeven de DEC vragen schriftelijk te beantwoorden en de aanvraag aan te passen. Deze is op 05-10-2023 ontvangen en besproken in de vergadering van 12 oktober 2023.**

Inhoudelijk heeft het beoogde project veel discussie opgeleverd t.a.v. de strategie, go/no go criteria, de 3 V's, de navolgbaarheid van de lotgevallen van de individuele dieren en het aantal benodigde dieren. Enkele van bovengenoemde punten zijn opgelost.

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

8. Eventueel horen van aanvrager
   - Datum: **17-08-2023**
   - Plaats: 5.1 lid2h
   - Aantal aanwezige DEC-leden: **6**
   - Aanwezige (namens) aanvrager: **1**
   - Gestelde vraag/vragen: **zie hieronder**
   - Datum antwoord: **05-10-2023**
   - Verstrekt(e) antwoord(en): **zie hieronder in blauw.**
   - De antwoorden hebben **wel** geleid tot aanpassing van de aanvraag

Vragen en *antwoorden*

1. You propose to conduct this research using rats, with genotypic selection based on serotonin transporter (SERT) polymorphisms. Extensive research since the landmark human study by Caspi et al. in 2003 has shown a significant correlation between the S allele and lifetime prevalence of depressive symptom components. You refer to Tiemeier et al.'s 2012 study for the human context and justify your study by stating that "no one has studied whether the onset of depression in adult offspring is mediated by the induction of cumulative early life stress exposure and the SERT genotype." However, upon review and interpretation by the 5.1 lid2h (Institutional Animal Care and Use Committee) of recent literature on cumulative stress factors before, during, and after pregnancy and their connection to later-life depressive symptoms, we revealed some evidence in humans. This is drawn from Guardino et al., Dev Psychobiol 2022, and Delli Colli et al., Translational Psychiatry 2022, stating:
   - "The 5-HTTLPR x stress interaction is a dynamic process and produces different effects at different time points, and indirectly confirm that s-allele carriers are both at higher risk and more capable to recover from depression."
   - "Expanding interplay between 5-HTTLPR and stress adding the temporal dimension, results in a three-way interaction: gene x environment x time."

   Based on this, the 5.1 lid2h concludes that "The human literature comprises fairly conclusive data on vulnerability to development of depression later in life from observational studies with early life stress in pregestational, prenatal, and postnatal periods." This contrasts with your assertion that this aspect has not been sufficiently investigated.
   As such, we have substantial doubts about the "utility and necessity" of the extensive animal study you propose. This constitutes a fundamental consideration in granting permission for animal studies under the Animal Experiments Act. We kindly request additional arguments beyond those provided in the research proposal to convince us of the adequacy of the study's utility and necessity.
   In the CCD project proposal, we clearly stated that there is an association between 5-HTTLPR and the onset of depression. In doing so, we also indicated that there are a number of studies that do not show this effect. Next, we describe the effects of ELS (early life stress) on mental health later in life. Here we describe that there are 3 types of ELS that have an effect on the mental health of the offspring 1) pregestational; 2) prenatal; and 3) postnatal (see background project proposal). What we next describe is that, in our opinion, no studies that investigate the interaction between cumulative early life stressors and the 5-HTTLPR have been performed.
   The 5.1 lid2h then describes that anno today there is sufficient scientific evidence in humans that these factors have also been studied cumulatively and bases itself mainly on the following articles:
   1) The 2022 study by Guardino et al. which shows that mothers who had PTSD before pregnancy have a higher risk of having children with a flatter diurnal cortisol slope
   2) The 2022 Delli Colli et al. study which conducted a systematic review with meta-analysis showing that in particular chronic stress interacts with the 5-HTTLPR which is only apparent when tested within a year of the stressor.

Our response to this is as follows:
Study 1 only studies the effects of pregestational stress on cortisol levels. No cumulative effect was measured here. Actually PTSD, depression and stress before pregnancy of a second child (measured after the delivery of a first child) was studied. Despite not ruling out the possibility that the effects are long-lasting and also play a role during and after pregnancy, this was not measured in this study. In addition, this study did not include the interaction with the 5-HTTLPR. Whereas we are precisely interested in this interaction with ELS. Also, this study did not study depressive symptoms, but physiological changes (cortisol). Certainly, an important finding, but in our opinion adds nothing to our previous reference that we have already cited showing that pregestational stress has a positive association with depression in offspring (systematic review with meta-analysis of Su et al 2021).
Study 2 shows very nicely that there is an interaction between the 5-HTTLPR and chronic stress. And that this is only significant when depression is measured within a year after the stressor has ended. A very important finding that, as the 5.1 lid2h *concludes, shows that there is a "gene x environment x time" interaction. However, the "time" referred to here is not the same as the time referred to by us, which is "early in life." Despite the interesting fact that s-allele carriers recover faster and recover in the longer term from their increased risk of depression, this article does not mention that the acute stress studies included in the meta-analysis did not include "early life stress." This means that acute stress during adult life has no long-term effect on depression (regardless of 5-HTTLPR), however it remains unclear whether it does when the stressors take place early in life. In addition, the effects of cumulative acute stressors were not examined, so we still do not know how that interaction with the 5-HTTLPR occurs. Nine out of the thirteen chronic stress studies included in the meta-analysis, included stress "early in life". While in the acute studies none of them included early life stress. Therefore, the question that remains unanswered is whether this effect is significant due to the timing, i.e., early in life, that these chronic stressors took place or due to their chronic duration (or both). This meta-analysis study does not answer this, and it does not even discuss this option.*
We also want to emphasize that in our proposal we will study the underlying mechanisms and we will study the transgenerational effects after cumulative ELS (interacting with the 5-HTTLPR). Something that has not been studied in humans.
With this, we hope the 5.1 lid2h *recognizes that the human literature is unfortunately not "fairly conclusive" about the susceptibility of developing depression later in life as a result of cumulative early life stressors (pregestational, prenatal and postnatal) in interaction with the 5-HTTLPR and sees the utility and necessity of the study we propose in the CCD application.*
We added the studies that the 5.1 lid2h *mentioned to the introduction of the project proposal. We also explain there why these studies are still inconclusive in answering our question.*

2. Following your reasoning that "There are ethical and methodological limitations to control the timeframe and type of cumulative early life stress exposure that leads to elucidate its role in the adult onset of depression in S-allele carriers" (in humans) and "In summary, it is critical to get fundamental insight in how SERT and ELS interact to induce stress related disorders, not only to understand the mechanisms underlying these vulnerabilities," you assert that the described research aims "to pave the way for further research into new treatment targets of (maternal) depression." Could you elaborate on how this animal research should indicate new drug targets, considering the molecular differences between human 5-HTTLPR and SERT and those in rats (in other words, the presence of a 'valid animal model')? (Page 3, 1st line)
In humans, a functional promoter polymorphism (5-HTTLPR) exist that modulates SERT expression. The short variant (s-allele) results in reduced expression of SERT and mRNA, less membrane-bound SERT and less 5-HT uptake. The heterozygous SERT-ko is an ideally suited model for the human s-allele 5-HTTLPR because here a proportion of the SERT gene is deleted resulting in less SERT protein expression and reduced 5-HT uptake. In both the human s-allele studies and the heterozygous SERT ko rats, there is no difference in basal

5-HT levels.
The reason why we think possible new treatment targets could be found is because the interaction between cumulative ELS and reduced SERT expression could lead to, for example, modulatory changes of neuronal firing properties, signaling processes, long-term synaptic plasticity and neurogenesis. Currently, selective serotonin reuptake inhibitors in particular are used to increase neurogenesis, for example, but if neurogenesis processes are found to underlie the ELS x SERT interaction, other substances that increase neurogenesis could be tested (e.g. psychedelics, or other, novel, targets). The main goal here is not to develop new targets, the goal is to understand what mechanisms play a role. Once that is known, these targets can be addressed (in future studies).

3. The project's background information seems to lack recent literature. Could you incorporate more recent references? In addition, can you precisely clarify how the present study relates to or follows from findings in previous studies where early life stress was studied against the background of the offspring's SERT genotype in males and females (5.1 lid2e, 5.1 lid2h 2020).
   We used literature that support our text and we are of the opinion that the literature we used is still highly valid and not outdated. We also used seminal papers. Newer literature available adds to the literature we already described, but did not bring new insights that changed the point of view. Nevertheless, in the revised version we included more publications of the last five years (including our own papers).
   In light of our previous experiments where dams were subjected to early life stress (ELSD) and their offspring behaviour was tested, we saw that in SERT+/- male offspring showed reduced anxiety and depressive-like behaviour (5.1 lid2e, 5.1 lid2h 2020), an effect not found in females, nor in SERT+/+ rats. This implicates a higher sensitivity in SERT+/- rats to pre-gestational stress, but outcomes are beneficial. Regarding social behaviours, we found no ELSD x genotype effects, although SERT+/- offspring in general engaged less in social interaction (5.1 lid2e, 5.1 lid2h 2019). An ELSD x genotype interaction was found in aggressive behaviour, with SERT+/- rats showing increased offensive behaviours compared to their controls (5.1 lid2e, 5.1 lid2h 2020). One explanation not finding large effects in affective behaviour could be explained by the fact that the ELSD we applied did not induce robust depressive-like behaviour in the mother. In our first study we found lower sucrose preference in females exposed to the early life stressor (5.1 lid2e, 5.1 lid2h 2019), however, we couldn't replicate this effect (5.1 lid2e, 5.1 lid2h chapter 8, thesis). When testing the offspring of ELSD, we did not test the mothers for depressive-like behaviours. Therefore, it is hard to tell whether all mothers showed a depressive-like effect. Of interest though is the recent study of Woo et al., 2023 who showed similar effects after pregestational stress in SERT+/- mice. When chronic variable stress was applied during pregnancy, male offspring showed reduced anxiety levels, but also reduced social preference. This study, together with our studies, points to the direction that males might be more vulnerable to ELS in respect to social behaviour aspects, but less to the affective behaviour aspects. It is therefore important to test both of these behaviours in both sexes.

4. Are there responders / non-responders in the model, in other words, should we assume that only animals responding to the stress protocol will be further included? Please provide your perspective on this.
   We will use all animals as we hope that this stress model will introduce robust effects. It might happen that there are non-responders, but we will not select the animals. In the transgenerational studies (phase 2; study 5) we will confirm depressive-like phenotypes, however we still plan to take along all mothers, as stress might have an effect on the next generation, even without a phenotype in the mother.

5. Why do you intend to use wildtype rats when there was no effect observed in heterozygote rats, which serve as models for genetic polymorphism in humans? Would this then become yet another multi-hit depression model?
   We expect the $SERT^{+/-}$ rats to be more vulnerable to the effects of ELS than $SERT^{+/+}$ rats. However, when it turns out that $SERT^{+/-}$ and $SERT^{+/+}$ rats respond similar, and there is no

difference between the two genotypes, we want to respect the 3 R's. That is, reducing the number of animals to the absolute minimum. When there is no difference between SERT$^{+/-}$ and SERT$^{+/+}$ rats in response to ELS, we will stop the breeding of SERT rats for this purpose, which will save breeding surplus. We will only use this cumulative ELS model when it turns out to be effective. That is the reason why we first apply a pilot study (as discussed in the DEC meeting). If the cumulative stressors do not have an effect, we will not continue with this (multi-hit) model. If there is a robust effect, but no difference in genotypes, we will continue with the SERT$^{+/+}$ to reduce breeding surplus animals, because we are interested in the underlying mechanisms of ELS in the development of depression and SERT$^{+/+}$ rats can provide highly valuable information.

6. How frequently do intense stressors like forced swimming in cold water occur in the Unpredictable Stress Protocol? This information is necessary for accurately assessing animal discomfort.
   The forced swim test will be applied 6 times in 21 days. We consider this as moderate stress based on our previous experience in a pilot experiment, where we exposed mothers to stress while their pups were maternally separated. In that study, mothers were 7 times in 14 days exposed to the forced swim test. At no point did we assessed this as a different discomfort scale than moderate.

7. The explanation in the appendix regarding cumulative discomfort is not sufficiently clear. In this argumentation, you should demonstrate that the cumulative discomfort remains within the moderate level. Furthermore, it is important to emphasize that a thorough evaluation of discomfort scores will take place during the initial phase, in close consultation with the IACUC (Institutional Animal Care and Use Committee). In this section, well-informed decisions can also be made regarding the continuation or termination of the research, based on solid reasoning.
   We added the text below to the appendix. We hope it is clearer how we estimated the cumulative discomfort levels to 'moderate'. We realize that the experiments might be on the high end of the moderate discomfort levels, but still expect them within those levels.
   For the pilot study, we expect the pregestational, prenatal and postnatal stressors to be in the range of moderate. Especially because in our hands, the pre- and postnatal stressors have never led to a higher discomfort than 'moderate'. The combination of all three stressors is new, and we will monitor the animals closely and will contact immediately the Institutional Animal Care and Use Committee when the discomfort will be higher than anticipated (or when in doubt). Experiments will end (in consultation with the IACUC) when they exceed "moderate" discomfort levels. The animal behavior tests that the animals will undergo, are similar to what we have performed before in our lab and are in the range of mild. Cumulative discomfort for this group will therefore be estimated as 'moderate'. For all other groups, the behavioral tests we will use are tests we have ample experience with. In our hands, they never exceeded 'moderate' discomfort levels. The early life stressor with the most robust effects will be used, which may mean the pregestational, prenatal and postnatal stressors might be picked as the preferred model. We expect the discomfort of the mother to have an impact on the offspring, however we still expect this to be in the 'moderate' range when combined with the behavioral experiments (which are mild to moderate).

8. Are the utility and necessity still proportionate? In other words, if such an intense model needs to be developed, which likely falls toward the higher end of the moderate scale and may involve severe distress, does this still align with the potential benefits (such as testing new medications)? Is it realistic for such an intense model to be employed for such purposes? Please provide your perspective on this.
   Understanding the biological mechanisms of depression is crucial for developing effective treatments. To unravel underlying mechanisms contributing to depression we are in need of a translational animal model. Our SERT$^{+/-}$ rats show translational value as they, like the s-allele carriers of the 5-HTTLPR in humans, have reduced serotonin reuptake (see also response to question 2). We know that ELS has an impact, and cumulative stressors seem

to more robustly affect s-allele carriers. When we understand the underlying mechanisms, we can start developing proper treatment for those targets. In our opinion this does outweigh the potential benefits of the moderate discomfort we expect the animals to undergo.

9. During the discussion, you agreed with a proposal by the DEC to introduce an additional phase (Phase 1) into the study. In this phase, an evaluation of the most intense model will be conducted based on crucial depression parameters like anhedonia, anxiety, and cognition.
   Because it is unclear whether the stressors applied will have the robustness of inducing depressive-like phenotype, the DEC suggested to first perform a pilot study. We can agree to this, and therefore implemented this pilot study in the revised version of the appendix. In this pilot, we will apply the most robust ELS, including pregestational, prenatal and postnatal stressors. The offspring will be tested for anxiety, anhedonia, cognition, and social behavior. We will have a go, no-go moment after this pilot, where we will only continue when we find a behavioral effect in our offspring.

10. The disparity between the English text and the NTS is substantial. Could you align them more closely for better consistency and clarity?
    We adjusted the NTS to align it more to the English text.

11. A considerable number of diverse tests will be conducted on the animals. In light of the 3Rs principles (Replace, Reduce, Refine), would it be possible to consider streamlining these tests into a more manageable selection? We would appreciate your perspective on this matter.
    We want to have a good overview of several depression-related behaviors and have kept it to a minimum to be able to gain insight into affective behavior, cognitive ability, and social behavior. All are observational studies, not invasive studies. We have done such studies before (with even more experiments (see thesis Houwing). From our experience we know that animals are well able to deal with this number of experiments because of the observational character. Performing these tests will provide us a better overall picture of the affected behaviors, especially as males and females might respond differently to the different behavioral tests. After the initial studies, we will zoom in on certain behaviors (as we do in phase 2 experiment 2-4).

12. The text in 3.3.2 concerning the stakeholders of this project appears incomplete. Why have patients with depression not been included as stakeholders, considering that this is a translational research study?
    Patients with depression are definitively stakeholders in the long-term. We added them to the 3.3.2 section of the revised version of the project proposal.

13. Could you provide more concrete descriptions of the specific outcomes expected upon reaching the milestones in Section 3.4.1, enhancing the connection with the intended project objectives?
    We adjusted the milestones in the revised version of the Project proposal. We hope they are more concrete now and linked to the intended project objectives.

14. To assess the realism of the quantity calculations, it would be desirable to include the outcome parameters and effect sizes used for each study. Can you add this?
    We have added this information to the revised version of the appendix.

15. The title in the 'administrative' form differs from the title of the project proposal. It is proposed using the latter, more concise title in the administrative form as well. There is also a discrepancy between the NTS (Dutch acronym for Project Title) in the administrative form and the actual NTS title. It is suggested to align the administrative form's NTS title with the actual NTS title as well. Could you align them for better consistency and clarity?
    We adjusted the administrative form and have aligned the proposal title and the NTS title.

9. Correspondentie met de aanvrager
N.v.t.

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.

Het project is vergunningplichtig

2. De aanvraag betreft een nieuwe aanvraag / een wijziging op een bestaande vergunning.

Nieuwe aanvraag zie A4

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.

N.v.t.

C. Beoordeling (inhoud)

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

Het hoofddoel van dit project is om te onderzoeken of de interactie van zogenaamde cumulatieve early life stress (ELS) (stress voor, tijdens en net na de dracht) en verstoring in de serotonine huishouding door een genetische verandering in het heropname-mechanisme van serotonine leidt tot een grotere gevoeligheid voor het ontwikkelen van een depressief-achtig fenotype en wat de onderliggende moleculaire mechanismen hiervan zijn; verder, in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie.

Om dit hoofddoel te bereiken, worden zes studies uitgevoerd. In een pilotstudie wordt bepaald of bij ratten de gecombineerde blootstelling aan (maternale) pre-gestationele ELS, (maternale) prenatale ELS en postnatale ELS een depressief-achtig gedragsfenotype induceert. Daarnaast wordt bepaald of het hebben van een serotonine re-uptake transporter (SERT) +/- genotype het risico op depressief gedrag verhoogt na de blootstelling aan cumulatieve ELS. In de vervolgstudies 1 tot en met 5 wordt onderzocht: 1) welke neurobiologische

mechanismen betrokken zijn bij het depressief-achtige fenotype geïnduceerd door cumulatieve ELS; 2) of het sociaal functioneren en de neurobiologische mechanismen ervan zijn veranderd bij jonge en volwassen ratten, die worden blootgesteld aan cumulatieve ELS; 3) in hoeverre het depressief-achtige fenotype geïnduceerd door cumulatieve ELS in de ene generatie wordt overgedragen op de volgende generatie en wat de betrokken neurobiologische mechanismen zijn.

Achtergrond, doelstelling en uitkomstparameters zijn duidelijk beschreven. De samenhang tussen de experimenten in de context van de doelstelling is helder en navolgbaar beschreven.

Echter, de strategie is niet duidelijk genoeg beschreven. De criteria voor het go/no go beslismoment na het pilot-experiment zijn niet duidelijk vastgesteld. Het is niet duidelijk welke randvoorwaarden er nodig zijn om met de vervolg experimenten verder te gaan. Er staat bijvoorbeeld niet of een significante verandering in één of meerdere van de 7 gedragstesten nodig zijn om verder te gaan. Of de responders en non-responders worden meegenomen is evenmin duidelijk, alsmede hoe er wordt omgegaan met de verschillen tussen mannelijke en vrouwelijk dieren. Tevens is niet duidelijk of de studie beëindigd wordt (no go) wanneer de SERT +/- ratten geen additioneel verschil laten zien.

5.1 lid2h **is op grond van bovenstaande van mening dat dit project toetsbaar is, overeenkomstig voorbeeld 1A van de handreiking 'Invulling definitie project'.**

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).

De 5.1 lid2h **heeft hier geen onderzoek naar ingesteld, maar voor zover de** 5.1 lid2h **kan beoordelen is er geen mogelijk tegenstrijdige wetgeving die uitvoering van het project in de weg kan staan, dit op basis van de beschikbare informatie uit het aanvraagformulier. De aanvrager geeft onder punt 3.2.3. van het project voorstel formulier ook aan dat er geen sprake is van tegenstrijdige wetgeving die het uitvoeren van de proef in de weg zou kunnen staan.**

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.

De classificaties als 'Fundamenteel onderzoek' en 'Translationeel of toegepast onderzoek' zijn verdedigbaar en acceptabel voor de 5.1 lid2h

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 Praktischehandreiking ETK: Stap 1.C4; zie bijlage I voor voorbeeld)*.

Het directe doel van het project is om een proefdiermodel te ontwikkelen dat depressieve symptomen vertoont na cumulatieve Early Life Stress in samenhang met een aanpassing van de activiteit van het SERT-gen om daarmee de

betrokken neurobiologische mechanismen te onderzoeken. Verder, in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie. Het uiteindelijke doel is om met de kennis van de neurobiologische mechanismen die betrokken zijn bij het (ontstaan van het) depressief-achtige fenotype in de toekomst nieuwe behandelingen voor depressie, vooral bij (aanstaande) moeders, te ontwikkelen.

Er is een directe relatie tussen het directe en uiteindelijke doel. Naar de mening van de 5.1 lid2h **zijn beide doelen gerechtvaardigd binnen de context van het onderzoeksveld.**

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 Praktische handreiking ETK: Stap 2.B en tabel 1; zie bijlage I voor voorbeeld)*

De belangrijkste belanghebbenden in dit onderzoek zijn de proefdieren, de onderzoekers en depressieve patiënten.

Waarden die voor proefdieren – ratten - in het geding zijn: de dieren worden in hun integriteit en welzijn aangetast door middel van een genetische verandering (SERT +/- ratten), blootstelling aan herhaaldelijke (cumulatieve) stressoren (ELS) alsmede aan 'intruders', een zevental gedragstesten, anesthesie, herhaaldelijke s.c. injecties en bloedafnames, individuele huisvesting en leven met depressie-gerelateerde verschijnselen. De dieren zullen hiervan ongerief en stress ondervinden. Aan het eind van de proeven worden de dieren gedood.

Waarden die voor onderzoekers bevorderd worden zijn: het beschikken over een diermodel, dat depressieve symptomen vertoont, veroorzaakt door cumulatieve Early Life Stress en verstoring in de serotonine huishouding door een genetische verandering in het heropname-mechanisme van serotonine om de neurobiologische mechanismen die betrokken zijn bij een depressief-achtig fenotype te kunnen bestuderen in de rat; verder, in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie..

Waarden die voor depressieve patiënten bevorderd worden zijn: in de toekomst mogelijke nieuwe behandelmethoden tegen depressie.

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

Nee, er zijn in deze aanvraag geen aanwijzingen die aanleiding geven om effecten op het milieu te verwachten.

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 Praktische handreiking ETK: Stap 1.C5).*

De 5.1 lid2h **is bekend met de kennis en ervaring van de onderzoeksgroep met het bestuderen van ELS-effecten op de hersenontwikkeling en het gedrag van diermodellen en het uitvoeren van gedragsexperimenten om cognitie, affectief gerelateerd en sociaal gedrag bij knaagdieren te beoordelen, ook bij serotonine re-uptake transporter knockout dieren (SERT +/-). Daarnaast heeft de**

onderzoeksgroep ervaring met het uitvoeren van relevante moleculaire analyses en is de benodigde infrastructuur beschikbaar bij het instituut waartoe de onderzoeksgroep behoort.

Naar mening van de 5.1 lid2h zijn hiermee de kennis en kunde van de aanvrager voldoende gewaarborgd.

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 Praktische handreiking ETK: Stap 1.C6)*.

Bij C1 is de opzet van het project in verkorte vorm weergegeven. Voor dit project is het huidige diermodel op wetenschappelijke en praktische gronden gekozen. De opzet en uitkomstparameters sluiten aan bij de gestelde doelen.

Echter, de strategie is hierbij niet geheel helder en/of logisch. Het is niet duidelijk voor welke uitkomstparameters het noodzakelijk is om een significant en robuust verschil te laten zien tussen experimentele groepen in de pilotstudie om daarna een vervolgstudie te gaan doen. Er is bijvoorbeeld niet beschreven of een enkele specifieke uitkomstparameter (zoals social play) voldoende is of dat één uit elke categorie voldoende is om verder te gaan.

Met deze opzet is er wel een gerede kans dat binnen de looptijd van het project progressie gemaakt zal worden in het ontwikkelen van een diermodel dat een of meer depressieve symptomen vertoont na cumulatieve Early Life Stress in samenhang met een aanpassing van het SERT-gen. Echter of, en in welke mate, de nieuw verkregen kennis in dit ratmodel zal leiden tot het ontdekken van nieuwe medicijnen is de 5.1 lid2h niet geheel duidelijk.

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 Praktische handreiking ETK: Stap 1.C1; zie bijlage I voor toelichting en voorbeelden).*

N.v.t.

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.

De aanvrager heeft aangegeven dat verzorging en huisvesting van de dieren conform de richtlijn zullen zijn. Echter, dieren die in studie 3 een 'resident-intruder' test ondergaan, zullen 21 dagen individueel worden gehuisvest. Dit is uitvoerig beschreven en voor deze experimenten noodzakelijk.

Het individueel huisvesten van de dieren leidt tot matig ongerief in ratten en is naar mening van de 5.1 lid2h voldoende onderbouwd.

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

Het is niet duidelijk of er bij deze aanvraag in rubriek F van de bijlage een realistische inschatting is gegeven voor het cumulatieve ongerief als maximaal licht voor 22% van de dieren en maximaal matig voor 78% van de dieren. Voor de pilotstudie is als inschatting gegeven voor het cumulatieve ongerief zijnde maximaal matig voor 100% van de dieren.

Het is volgens de 5.1 lid2h niet uit te sluiten dat er in de uiteindelijke proeven combinaties van handelingen zullen worden gebruikt die leiden tot een stapeling van ongerief op een zodanige wijze dat het in de bijlage beschreven cumulatieve ongerief van matig wordt overschreden. Dit geldt voor zowel de pilotstudie als alle vervolgstudies. De beschrijving van de procedures geeft een onvolledig overzicht en omdat er niet beschreven is hoe het maximaal cumulatief matig ongerief wordt gewaarborgd, acht de 5.1 lid2h de inschattingen van het (cumulatieve) ongerief twijfelachtig en mogelijk te laag.

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 Praktische handreiking ETK: Stap 1.C2). (zie bijlage I voor voorbeeld).*

De integriteit van de dieren zal worden aangetast door middel van een genetische verandering (SERT +/- ratten), blootstelling aan herhaaldelijke (cumulatieve) stressoren (ELS) alsmede aan 'intruders', een zevental gedragstesten, anesthesie, herhaaldelijke s.c. injecties en bloedafnames, individuele huisvesting en leven met depressie-gerelateerde verschijnselen. De dieren zullen hiervan ongerief en stress ondervinden. Aan het eind van de proeven worden de dieren gedood.

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 Praktische handreiking ETK: Stap 1.C3)*.

3V's

De omstandigheden waaronder humane eindpunten worden bereikt worden in de bijlages onder E beschreven. De aanvrager beschrijft dat de waarschijnlijke incidentie erg laag zal zijn.

De aanvragers hebben de humane eindpunten geformuleerd. Dit zijn:
- **Gewichtsverlies van meer dan 15% binnen 48 uur.**
- **Duidelijke tekenen van ziektegedrag vertonen en/of grimassen vertonen (gebogen rug, ruwe vacht, algemene malaise).**

Op grond van het bovenstaande meent de DEC dat de humane eindpunten goed zijn ingeschat en beschreven.

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

In dit onderzoek wordt een proefdiermodel ontwikkeld voor depressie als gevolg van Early Life Stress in samenhang met een verminderde functie van het SERT-gen. De uitkomstparameters die worden gemeten - gedrag en neurobiologische processen- zijn complexe mechanismen, die een levend dier vereisen om de cumulatieve ELS-effecten op de hersenen te kunnen waarnemen. Ratten zijn in staat een breed scala aan gedragingen te vertonen die functioneel vergelijkbaar zijn met die bij de mens. Tot op heden zijn er nog geen proefdiervrije alternatieven ontwikkeld om gedragspatronen te bestuderen.

De 5.1 lid2h **is van mening dat de aanvrager voldoende aannemelijk heeft gemaakt dat er geen vervangingsalternatieven zijn.**

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)*.

Het aantal dieren dat nodig is voor de pilotstudies is zorgvuldig overwogen op basis van eerdere studies en de verwachte variantie in de afhankelijke variabelen.

Als de bevindingen van de pilotstudie geen gedragsmatige primaire uitkomsten van een depressief-achtig fenotype laten zien bij ratten, die zijn blootgesteld aan cumulatieve ELS, zal het uitvoeren van fase 2 niet langer relevant zijn. Bovendien, als SERT +/- ratten geen hogere kwetsbaarheid voor depressief gedrag vertonen dan SERT +/+ ratten, zullen ze niet langer worden gebruikt in studies van fase 2. Daarom zullen alleen SERT +/+ ratten worden opgenomen en zou het aantal groepen en dieren in studies 1 tot 5 worden verminderd.

Het is, volgens de aanvrager, van cruciaal belang om fundamenteel inzicht te krijgen in hoe ELS en SERT op elkaar inwerken om stress-gerelateerde stoornissen te induceren. Het is de 5.1 lid2h **daarom niet duidelijk waarom het uitvoeren van fase 2 nog langer relevant is als SERT +/- ratten geen hogere kwetsbaarheid voor depressief gedrag vertonen dan SERT +/+ ratten en vindt dat fase 2 dan ook niet moet worden uitgevoerd in dit geval.**

Het geschatte aantal dieren per groep per onderzoek is gebaseerd op statistische methoden die erop gericht zijn het aantal dieren tot een minimum te beperken en komen de nakomelingen van studies 2 tot 4 van dezelfde ouders, waardoor het gebruik van de nakomelingen wordt gemaximaliseerd.

De go/no go strategie en de opbouw van experimentele groepen zijn niet geheel helder. Echter, op basis van de beschrijving in de aanvraag is de 5.1 lid2h **van mening dat het aantal te gebruiken dieren realistisch is ingeschat en dat er voldoende getracht is om met zo min mogelijk dieren tot een betrouwbaar resultaat te komen. Het totale aantal ratten voor een periode van 5 jaar is daarmee een realistische en haalbare schatting op projectniveau.**

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 Praktische handreiking ETK: Stap 1.C3)*.

Alle genoemde procedures worden regelmatig door de onderzoeksgroep gebruikt en zijn eerder verfijnd om potentiële ongemakken te minimaliseren. Alle chirurgische ingrepen worden onder narcose uitgevoerd, met de juiste postoperatieve zorg. De kooien worden verrijkt met o.a. nestmateriaal, knaagstokjes en de dieren worden gehouden in groepshuisvesting. De ratten worden regelmatig gemonitord op welzijn en de dieren worden op humane wijze gedood op gedefinieerde eindpunten volgens nationale ethische regels. Alleen voldoende opgeleid en bekwaam personeel zal de dieren behandelen en alle procedures uitvoeren.

Echter, de onderzoeker geeft bij de monitoring van de procedures, zoals het blootstellen aan cumulatieve ELS, niet aan hoe het minimaliseren van het ongerief wordt gewaarborgd.

De onderzoeker beschrijft dat de handelingen zo worden uitgevoerd dat overbodige stress bij de dieren wordt voorkomen en het leed beperkt blijft tot maximaal cumulatief licht ongerief voor 22% van de dieren en verwacht maximaal cumulatief matig ongerief voor 78% van de dieren. Echter gezien de aard en intensiteit van de individuele stressoren die cumulatief worden aangeboden aan de proefdieren is de 5.1 lid2h **van mening dat er onvoldoende waarborgen zijn voor beperking van ongerief om deze procedure vrij te geven voor een project van deze omvang en het door de onderzoekers ingeschatte cumulatieve ongerief van maximaal matig voor de ruime meerderheid van de dieren.**

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.

N.v.t.: Het betreft 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 Praktische handreiking ETK: Stap 1.C3; zie bijlage I voor voorbeeld).*

De aanvrager geeft aan zowel mannelijke als vrouwelijk dieren te willen gebruiken.

Depressie bij mensen komt vaker voor bij vrouwen dan bij mannen (zie de aanvraag). In de rattenproeven door de groep gedaan (zie aanvraag en antwoorden hierboven) lijken er onderscheidenlijke effecten te zijn tussen mannelijke en vrouwelijke dieren, die niet één-op-één vertaalbaar lijken te zijn naar die bij mensen. Het onderzoek wordt uitgevoerd bij zowel mannelijke als vrouwelijke ratten om meer helderheid hierover te krijgen.

De 5.1 lid2h is van mening dat de aanvrager dit punt in voldoende mate wetenschappelijk heeft onderbouwd.

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 Praktische handreiking ETK: Stap 1.C3).*

De meeste dieren worden gedood in het kader van het project voor de isolatie van hersenen en andere weefsels, die benodigd zijn voor mechanistisch onderzoek.

Voor de pilotstudie en studies 1 en 4 worden de dieren verdoofd door inademing van CO2 en zo snel mogelijk onthoofd.

Voor studies 2 en 3 worden de dieren gedood door een letale overdosis anesthesie, d.m.v. een intra-peritoneale toediening van pentobarbital.

Ten aanzien van studie 5:

- **Matig-gestreste F1-dieren in studie 5 zullen worden gedood na het voltooien van de gedragstests en/of het fokken, omdat ze het wetenschappelijke doel hebben bereikt. De manier waarop dat wordt gedaan is niet beschreven.**
- **De F2 dieren van studie 5 zullen worden gedood in het kader van het project voor de isolatie van hersenen. De dieren zullen worden verdoofd door inademing van CO2 en zo snel mogelijk worden onthoofd.**

Ondanks dat niet van alle dieren de dodingmethode volledig is beschreven, gaat de 5.1 lid2h ervan uit dat alle dieren worden gedood door een voor de rat passende dodingsmethode conform bijlage IV van richtlijn 2010/63/EU.

De 5.1 lid2h is van mening dat de aanvrager dit punt in voldoende mate wetenschappelijk heeft onderbouwd. Zie bijlage 1 punt K.

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

In studie 5 kunnen niet-gestreste F1-ratten worden aangeboden voor hergebruik voor educatieve doeleinden, omdat ongemak bij deze dieren als mild wordt geclassificeerd. Als ze echter door de aangewezen dierenarts ongeschikt worden verklaard voor verder gebruik, worden ze gedood volgens de EU-richtlijn (deze methode wordt echter niet beschreven). Dieren worden alleen voor hergebruik aangeboden als ze aan het einde van het onderzoek geschikt zijn voor andere experimenten, die onder een bestaande CCD-vergunning vallen of studies die onder de drempelwaarde liggen. Om hoeveel eventueel te hergebruiken dieren het gaat is niet beschreven in de bijlage noch in de NTS.

NTS

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

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

D. Ethische afweging

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

In dit advies worden twee morele vragen beantwoord. De 5.1 lid2h **koppelt de pilotstudie los van de rest van de studie:**

1) **Rechtvaardigen de doelstellingen van het project '*Transgenerational susceptibility to depression due to early life stress*' het cumulatief maximaal licht (22%) tot matig (78%) ongerief dat de proefdieren wordt aangedaan in het onderhavige project?**
2) **Rechtvaardigen de doelstellingen van de pilotstudie van het project '*Transgenerational susceptibility to depression due to early life stress*' het cumulatief maximaal matig (100%) ongerief dat de proefdieren wordt aangedaan in het onderhavige project?**

Zoals onder C1 uiteengezet acht de 5.1 lid2h **de aanvraag toetsbaar op project niveau en een ethische afweging is daarom mogelijk.**

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 belangrijkstebelanghebbenden 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 Praktische handreiking ETK: Stap 3.B; zie bijlage I voor voorbeelden).*

***Waarden die voor de proefdieren in het geding zijn:* de integriteit en het welzijn van de dieren zullen worden aangetast door een genetische verandering (SERT +/- ratten), blootstelling aan herhaaldelijke (cumulatieve) stressoren (ELS) alsmede aan 'intruders', een zevental gedragstesten, anesthesie, herhaaldelijke s.c. injecties en bloedafnames, individuele huisvesting en leven met aan depressie gerelateerde verschijnselen. De dieren zullen hiervan ongerief en stress ondervinden. Aan het eind van de proeven worden (de meeste zo niet alle) de dieren gedood.**

***Algemeen: w*aarden die voor onderzoekers bevorderd worden zijn het beschikken over een diermodel dat depressief-achtige symptomen vertoont veroorzaakt door cumulatieve Early Life Stress en verstoring in de serotonine huishouding door een genetische verandering in het heropname mechanisme van serotonine om de neurobiologische mechanismen die betrokken zijn bij een depressief-achtig fenotype te kunnen bestuderen in de rat; verder, in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie.**

Dit kan in de toekomst mogelijk resulteren in inzicht in de opbouw, ernst en karakteristieken van de door ELS opgebouwde depressie in samenhang met een verstoorde serotonine huishouding en eventueel aangrijpingspunten voor nieuwe behandelmethoden tegen deze vorm van depressie mogelijk maken.

De 5.1 lid2h **is van mening dat de belangen van de samenleving in het algemeen en de wetenschap in het bijzonder binnen de pilotstudie van het project '*Transgenerational susceptibility to depression due to early life stress*' zwaarder wegen dan de belangen/waarden van de dieren.**

Indien de doelstellingen van de pilotstudie bereikt worden, kan dit leiden tot de ontwikkeling van een diermodel dat later gebruikt kan worden voor de bestudering van cumulatieve ELS-effecten op depressief-achtig gedrag bij ratten, in samenhang met een verstoring in de serotonine huishouding door een genetische verandering in het heropname mechanisme van serotonine. Met dit diermodel kan in verder onderzoek ook bestudeerd worden in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie.

Vanuit dit perspectief is de 5.1 lid2h **van mening dat het onderhavige onderzoek wetenschappelijk van substantieel belang is. Het is aannemelijk dat de doelstellingen van deze pilotstudie behaald kunnen worden.**

Bij deze aanvraag heeft de 5.1 lid2h **onder andere gesproken over het nut en de noodzaak van het onderzoek, de strategie, go/no go criteria, de 3 V's, de intensiteit van de stressor behandelingen in relatie tot het mogelijk cumulatief ongerief, de navolgbaarheid van de lotgevallen van de individuele dieren en het aantal benodigde dieren.**

Met de antwoorden op de vragen van de 5.1 lid2h **en de gereviseerde versie van de aanvraag hebben de aanvragers deze (en andere punten) voldoende geadresseerd om de pilotstudie te vergunnen, maar heeft de** 5.1 lid2h **een probleem met het op voorhand verlenen van toestemming voor de pilotstudie in combinatie met de vervolgstudie om redenen van onduidelijkheden en onzekerheden in het protocol zoals boven genoemd.**

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 Praktische handreiking ETK: Stap 3.C; zie bijlage I voor voorbeeld)*.

1. De 5.1 lid2h **beantwoordt de centraal morele vraag: rechtvaardigt de doelstelling van het project '*Transgenerational susceptibility to depression due to early life stress*' dat is gericht op het verkrijgen van een nieuw diermodel om te kunnen onderzoeken welke neurobiologische mechanismen ten grondslag liggen aan de invloed van cumulatieve ELS op het serotoninesysteem dat een belangrijke rol speelt bij depressie het cumulatief maximaal licht (22%) tot matig (78%) ongerief voor de ratten in het voorliggende project *ontkennend*.**

2. De 5.1 lid2h **beantwoordt de centraal morele vraag: rechtvaardigt de doelstelling van de pilotstudie van het project '*Transgenerational susceptibility to depression due to early life stress*' dat is gericht op het verkrijgen van een**

nieuw diermodel dat cumulatieve ELS geïnduceerde depressieve verschijnselen vertoont in samenhang met een verstoring in de serotonine huishouding door een genetische verandering in het heropname mechanisme van serotonine het cumulatief maximaal matig (100%) ongerief dat de proefdieren wordt aangedaan in de pilotstudie *bevestigend*.

Hoewel de 5.1 lid2h de intrinsieke waarde van het dier onderschrijft en oog heeft voor het te ondergane ongerief van de proefdieren, weegt het potentiële substantiële belang van de pilotstudie in dit project naar haar mening zwaarder. De volgende overwegingen hebben bijgedragen tot deze conclusie:

- **Indien de doelstellingen van de pilotstudie bereikt worden, zal dit kunnen resulteren in een nieuw diermodel waarin door cumulatieve ELS geïnduceerde depressie-achtige gedragingen kunnen worden bestudeerd. Daarnaast wordt vastgesteld of het hebben van een serotonine transporter (SERT)+/- genotype het risico op depressief gedrag verhoogt na de blootstelling aan cumulatieve ELS; verder, kan daarna bestudeerd worden in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie.**
- **Het is voorstelbaar dat eventuele vervolgonderzoeken kunnen leiden tot wetenschappelijke kennis en inzichten op het gebied van moleculaire mechanismen, die ten grondslag liggen aan de invloed van cumulatieve ELS op het serotoninesysteem en daarmee op depressie-achtige gedragingen bij de rat wat vervolgens misschien kan leiden tot een beter begrip van hoe verschillende early life stressoren psychische veranderingen kunnen veroorzaken bij de mens.**

De 5.1 lid2h is van mening dat de gekozen strategie en experimentele aanpak van het project als geheel ten dele onlogisch overkomen en niet helder aansluiten bij de aangegeven doelstellingen.

De 5.1 lid2h is van mening dat de voorgestelde experimentele opzet van enkel de pilotstudie en betreffende uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstelling en naar verwachting zullen leiden tot het behalen van de doelstelling van de pilotstudie ter vaststelling van de onderzoeksparameters voor de vervolgstudies.

De onderzoekers beschikken over de benodigde kennis en technische expertise om het voorgestelde experimentele werk goed uit te voeren.

Om de doelstellingen van de pilotstudie te bereiken is het nodig om proefdieren te gebruiken. De onderzoekers schrijven dat ze er alles aan doen om het lijden van de ratten te beperken, waardoor het uiteindelijke (cumulatieve) ongerief van elk individueel dier, in de pilotstudie, naar verwachting beperkt blijft tot maximaal matig (100%). Er zijn naar mening van de 5.1 lid2h echter onvoldoende aanwijzingen om aan te nemen dat bij de uitvoering van de studies het maximale gedaan wordt om het cumulatieve ongerief te beperken tot maximaal matig, waardoor ernstig ongerief niet kan worden uitgesloten en zelfs kan worden verwacht in de pilotstudie.

Op grond van deze overwegingen beschouwt de 5.1 lid2h de voorgestelde dierproeven in uitsluitend de pilot van het projectvoorstel `*Transgenerational susceptibility to depression due to early life stress'* als ethisch gerechtvaardigd en voorziet de 5.1 lid2h derhalve enkel de pilotstudie van het onderhavige projectvoorstel van een positief advies en dientengevolge ontbeert de aanvraag voor alle overige onderdelen een (positief) advies.

E. Advies

1. Advies aan de CCD
   ☐ **De 5.1 lid2h adviseert uitsluitend voor de <u>pilotstudie</u> de vergunning te verlenen.**

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 Praktische handreiking ETK: Stap 4.A; zie bijlage I voor voorbeeld).*

De meningen van de 5.1 lid2h leden zijn verdeeld wat betreft de motivatie van de interventies, de logica van de vele interventies en of deze geminimaliseerd zijn. Het ongerief voor individuele dieren is redelijk duidelijk beschreven, maar niet dat er alles aan gedaan wordt om het maximale ongerief niet voorbij cumulatief matig te laten komen en ook niet hoe onnodig ongerief wordt voorkomen. De DEC-leden twijfelen mede daarom aan de correctheid van het te verwachten maximale cumulatief matig ongerief dat de aanvrager aangeeft.

Het advies is gebaseerd op een meerderheidsstandpunt.

☐ **Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten:**

De uitkomsten van de <u>pilotstudie</u> moeten gerapporteerd en beoordeeld worden. Daarin moet duidelijk worden dat:

1) **Het ongerief dat de dieren in de <u>pilotstudie</u> ervoeren cumulatief maximaal matig was,**
2) **Bij welke van de 7 gedragstesten er een significant en robuust effect te zien was door de specifieke (cumulatieve) ELS,**
3) **Dat SERT +/- ratten een hogere kwetsbaarheid voor depressief gedrag vertonen dan SERT +/+ ratten als gevolg van cumulatieve ELS,**
4) **De data verkregen van de responders en non-responders worden meegenomen in de uiteindelijke conclusies van het onderzoek; dit betekent dat van de vrouwtjes die gestrest zijn bepaald moet worden wat hun niveau van stress is; dit is dan een co-variant in de analyses van de nakomelingen; dit tegen de achtergrond van de eerdere studies van deze groep (zie de antwoorden hierboven).**

Twee DEC leden kwamen tot een negatief advies ten aanzien van de gehele aanvraag <u>inclusief de pilotstudie</u>. Deze leden zijn van mening dat voor dit project de belangen van de proefdieren, die in het geding zijn, zwaarder wegen dan de belangen van de overige belanghebbenden. De redenen hiervoor zijn:

- **Naar de mening van deze beide leden moet het blootstellen van de betreffende dieren aan drie zogenaamde early life stressors (ELS) zowel voor, tijdens als net na de dracht worden geclassificeerd als cumulatief ernstig ongerief, terwijl niet uitgesloten kan worden dat zulks voor een tweetal early life stressors ook geldt.**
- **Het twijfelachtig is of de resultaten van de studie bij de rat te extrapoleren zijn naar de mens daar de rat 5-HTTLPR niet tot expressie brengt.**
- **De potentiële opbrengst in de vorm van (nieuwe) farmaca ten behoeve van behandeling van depressie bij de mens onvoldoende wordt geduid.**
- **De lotgevallen van de individuele dieren nog steeds niet helder beschreven zijn.**

Concluderend: Op basis van de herziene aanvraag met de pilot en de beantwoording van de vragen zijn deze leden van mening dat er in de huidige versie onvoldoende grond is om te rechtvaardigen dat de doelstellingen van het project alsmede de voorgestelde en beargumenteerde uitvoering daarvan door de aanvrager zwaarder wegen dan het nu op de grens balanceren van cumulatief matig en ernstig ongerief van de ratten. Het voorgestelde project biedt in de huidige vorm onvoldoende perspectief voor nieuwe inzichten in de pathofysiologie en farmacotherapeutische behandeling van depressie bij mensen als gevolg van early life events in samenhang met variatie in de serotonine huishouding door een genetische verandering in het heropname-mechanisme van serotonine.

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 Praktische handreiking ETK: Stap 4.B)*.

Mogelijke onduidelijkheden en knelpunten zijn in twee vergaderingen besproken en ook met de onderzoekers gecommuniceerd tijdens een DEC-vergadering (zie vragen bij onderdelen A8 en de punten genoemd bij D. Ethische afweging). Naar het oordeel van de meerderheid van de 5.1 lid2h **zijn deze punten op bevredigende wijze opgehelderd voor het vergunnen van enkel de pilotstudie.**

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*
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
E-mailadres contactpersoon				
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		
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	5.1 lid2e	
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		
	E-mailadres		

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 - 09 - 2023	
	Einddatum (t/m)	31 - 08 - 2028	
3.2 Wat is de titel van het project?	Transgenerational susceptibility to depression due to early life stress		
3.3 Wat is de titel van de niet-technische samenvatting?	Transgenerationele gevoeligheid voor depressie door stress in het vroege leven		
3.4 Wat is de naam van de Dierexperimentencommissie (DEC) van voorkeur?	Naam DEC	5.1 lid2h	
	Postadres		
	E-mailadres		

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 1
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	5.1 lid2e
Plaats	5.1 lid2h
Datum	20 - 6 - 2023
Handtekening	5.1 lid2e

Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0800-7890789).

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.	Transgenerational susceptibility to depression due to early life stress

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.

Depression is a mental health condition characterised by sadness, loss of interest or pleasure, feelings of guilt and low self-worth that substantially impairs an individual's ability to cope with daily life. Cognitive impairments, altered affective behaviour, and social disfunctions are often present in depressed

individuals [1-3]. Depression is the most prevalent neuropsychiatric disorder and the first cause of disability in the world [4]. The social burden related to depression is steadily increasing, and the numbers for depressive disorders may increase even more in the next few years due to the unprecedented negative effects of the COVID-19 pandemic on mental health.

Women suffer from depression twice as often compared to men [5] and unfortunately pregnant women are not spared. Approximately one out of five pregnant women suffer from depressive symptoms [6], which can have long-term health consequences in the offspring [7]. Early life programming through an adverse intrauterine environment, or postnatally, increases susceptibility to a myriad of diseases, including psychiatric disorders [8]. Thus, an adverse early life environment increases the risk for depression. However, this risk is increased when a subject has vulnerable genes as was shown by the seminal paper of Caspi et al. [9]. This, and later studies identified the critical interaction of genetic and environmental factors that contribute to depression [10-13]. Caspi and colleagues found that the risk for depression increased when the number of stressful life events that people encountered increased. Additionally, they studied the extent to which the genotype that regulates the serotonin transporter (SERT) expression moderates the influence of stress on depression. In the serotonin-transporter-linked promoter region (5-HTTLPR), different lengths of the repetitive sequence containing GC-rich, 20-23-bp-long repeat elements in the upstream regulatory region of the gene have been identified. Deletion or insertion in the 5-HTTLPR is referred to as the 14-repeat short (S, low expressing) and the 16-repeat long (L, high expressing) alleles. Caspi and colleagues found that individuals who were carriers of one or two copies of the S-allele were more vulnerable to developing depression following stress exposure than carriers of the L-allele exposed to stress [9].

Many studies reproduced the Caspi's research, although a large genome-wide study found no association between the SERT genotype and increased risk for lifetime prevalence of depression in people exposed to stress [14]. Also, rodent studies fail to show solid evidence for increased vulnerability to developing depressive-like behaviour after early-life stress (ELS) in rodents with reduced SERT (heterozygous; $SERT^{+/-}$) expression [15]. Therefore, the potential association of the SERT gene and psychiatric condition remains inconclusive. Nevertheless, previous studies show that the influence of 5-HTTLPR can be heterogeneous and highlight possible involvement of other factors and regulatory mechanisms promoting the risk of psychiatric disorders [16,17]. Recently a study in an elderly Lithuanian population showed that a 5-HTTLPR × lifetime stressful events interaction effect on depression was observed [18]. The highest odds of depression were found in participants with both high stress and s/s genotype for life time stressful events.

What has been consistently shown is that ELS exposure has a large impact on mental health later in life [19,20], and that ELS is not restricted to stress exposure during childhood (postnatal stress), but can also include exposure to stress during the foetal period, mediated by a mother that is stressed during pregnancy or even before conception [21,22]. Considering pregestational stress, it was shown that physiological alterations (flattened child diurnal cortisol slopes) take place when posttraumatic stress disorder was diagnosed before pregnancy [23]. Thus, ELS to the offspring can take place by 1) maternal stress/depression before pregnancy (*pregestational*; which is transferred to the offspring during pregnancy); 2) maternal stress/depression during pregnancy (*prenatal*; transferred to the offspring during pregnancy); and 3) either maternal stress/depression, or direct stressors to the offspring after birth (*postnatal*; during the first years of life).

With this additional evidence of maternal stress mediating offspring long-term outcomes, the studies about the ELS x SERT genotype interaction can be revisited. One of the reasons that the ELS x SERT studies are inconclusive may be because sustained maternal stress, starting before child's birth and even before conception, has not been taken into account. Moreover, clinical research supports the notion that neuropsychiatric disorders, including depression, have a developmental origin mediated by the conditions of the mother before the conception of the child [21]. From this view, the exposure to *cumulative* ELS can interact with the SERT genotype -of the offspring- to increase the risk of depression. To our knowledge, no one has studied whether the onset of depression in the adult offspring is mediated by the interaction of *cumulative* ELS exposure and the SERT genotype. The study published by Tiemeier et al.

[24] strongly suggest the importance to do so. They found that maternal anxiety -that involves stress in the mother- during pregnancy and postnatally increased the risk of child emotional problems and leads to less accurate emotional matching in 3-year-old S-allele carriers. Of interest is the systematic review and meta-analysis of Delli Colli and colleagues who show that a 5-HTTLPR gene x environment x time interaction exist [25]. When depression is measured within a year after chronic stress a significant interaction with the 5-HTTLPR and stress was found, an effect that was not found after an acute stressor. The effect was also only found when the subjects were tested within 1 year after the stressor, and not when the stressor happened before the last year. However, what the authors fail to mention is that in the acute stressor group only stressors during adulthood took place, while in the chronic stress group 9 out of the 13 included studies involve early life stress. The question that rises, is whether the significant effect was found due to timing, or due to the fact of early life stress (or both). This is still not clear, and needs clarification.

There are ethical and methodological limitations to control the timeframe and type of *cumulative* ELS exposure that leads to elucidate its role in the adult onset of depression in S-allele carriers. Fortunately, valid animal models can help to study this association. Rodents do not express the 5-HTTLPR; however, heterozygous SERT knockout rodents ($SERT^{+/-}$) show neurochemical similarities to human S-allele carriers [26,27]. From a previous research project of our group, we provided a comprehensive overview of the behavioural effects of ELS in $SERT^{+/-}$ rodents and the neurobiological mechanisms involved [15]. We found that studies of postnatal ELS in $SERT^{+/-}$ rodents failed to show solid evidence for increased vulnerability to a depressive-like phenotype when $SERT^{+/-}$ adult animals were tested. We also performed a study in $SERT^{+/-}$ female rats to test whether their exposure to postnatal ELS increased their vulnerability to depressive-like behaviours when they were adults. The average of $SERT^{+/-}$ females did not exhibit consistent behavioural changes of depressive-like responses after ELS, although some females exposed to postnatal ELS showed increased affective-related behaviours [28]. In light of our previous experiments where dams were subjected to early life stress (ELSD) and their offspring behaviour was tested, we saw that $SERT^{+/-}$ male offspring showed reduced anxiety and depressive-like behaviour [29], an effect not found in females, nor in $SERT^{+/+}$ rats. This implicates a higher sensitivity in $SERT^{+/-}$ rats to pre-gestational stress, but outcomes are beneficial. Regarding social behaviours, we found no ELSD x genotype effects, although $SERT^{+/-}$ offspring in general engaged less in social interaction [30]. An ELSD x genotype interaction was found in aggressive behaviour, with $SERT^{+/-}$ rats showing increased offensive behaviours compared to their controls [31]. One explanation for not finding large effects in affective behaviours could be explained by the fact that the ELSD we applied did not induce robust depressive-like behaviour in the mother. In our first study, we found a lower sucrose preference in females exposed to the early life stressor [32], however, we couldn't replicate this effect in a later study [28]. When testing the offspring of ELSD, we did not test the mothers for depressive-like behaviours. Therefore, it is hard to tell whether all mothers showed a depressive-like effect. Of interest though is the recent study of Woo et al., 2023 [33] who showed similar effects after pregestational stress in $SERT^{+/-}$ mice. When chronic variable stress was applied during pregnancy, male offspring showed reduced anxiety levels, but also reduced social preference. This study, together with our studies, points to the direction that males might be more vulnerable to ELS in respect to social aspects, but not the affective aspects of behaviour. It is therefore important to test both of these behaviours in both sexes.

Considering the findings of our previous research project, postnatal ELS is not optimal or severe enough to induce a depressive-like phenotype in $SERT^{+/-}$ rodent models. In the present research project, we will investigate whether $SERT^{+/-}$ male and female rats are more vulnerable to develop a depressive-like phenotype in adulthood following the exposure to *cumulative* ELS. Moreover, this project will help us understand the underlying mechanisms of SERT x *cumulative* ELS interactions in the onset of depression later in life.

Based on our findings and the literature, we propose to apply pre-gestational, prenatal and postnatal stressors and investigate how these stressors cumulatively affect wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ offspring (both male and female). Not only are we interested in the mechanisms underlying this multiple stressor system, but also whether the SERT gene expression level increases the vulnerability to

depression following *cumulative* ELS. This will prevent using a high number of animals in the future as we could select more animals expressing a depressive-like phenotype after a single manipulation of multiple ELS stressors and use this for instance as a model for maternal stress.

We will study cognitive, affective-related and social behaviours, as well as gene expression and epigenetic markers (study 1). We will also characterise the social functioning and neurobiological mechanisms involved in studies 2 to 4. Lastly, we will test whether the offspring of the depression-like parents also express a depressive-like phenotype, as well as the extent to which epigenetic markers are shared in both generations (study 5).

In summary, it is critical to get fundamental insight in how SERT and ELS interact to induce stress-related disorders, not only to understand the mechanisms underlying these vulnerabilities, but also to pave the way for further research into new treatment targets of (maternal) depression. Because of the mechanistic approach in this research this is considered basic research. We hypothesise that a cumulative history of ELS exposure will increase the risk of disrupted cognitive, affective-related, social behaviours in the offspring. In addition, we hypothesise that animals with a vulnerable genetic background ($SERT^{+/-}$ rats) will be more vulnerable to develop depressive-like behaviours (or in a more severe fashion) than those with a non-vulnerable genetic background. Finally, we hypothesise that the effects of stressful early-life experiences of parents will be transferred to their offspring through inheritance of epigenetic markers.

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 research is to determine whether the interaction of *cumulative* ELS and the SERT genotype increases the vulnerability to developing a depressive-like phenotype and to what extent the parental depressive-like phenotype is transferred to the next generation. Neurobiological mechanisms -including epigenetic markers-, depressive-like behaviours -including affective-related, cognitive and social behaviour- and transgenerational effects will be elucidated in the rat model.

To reach this ultimate goal, immediate goals are proposed to be attained by conducting six studies:

Phase 1: Pilot study
Goal 1: To determine whether the combined exposure to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS, ie *cumulative* ELS, induces a depressive-like behavioural phenotype in the rat.
Goal 2: To determine whether the $SERT^{+/-}$ genotype increases the risk for depressive-like behaviours after the exposure to *cumulative* ELS.

Phase 2: Studies 1 to 5
Goal 3: To assess the neurobiological mechanisms involved in the depressive-like phenotype induced by *cumulative* ELS.
Goal 4: To identify whether social functioning and its neurobiological mechanisms are altered in young and adult rats exposed to *cumulative* ELS.
Goal 5: To determine the extent to which the depression-like phenotype induced by *cumulative* ELS in one generation is transferred to the next and evaluate the neurobiological mechanisms involved.

3.2.2 Provide a justification for the project's feasibility.

Our research group has ample experience conducting behavioural experimentation to assess cognition, affective-related and social behaviours in rodents, especially also in serotonin transporter knockout animals (SERT). The SERT animals have been bred at our facility for many years, making the project

feasible. In addition, our research group has experience in performing molecular analyses (microarray, qPCR, genotyping, DNA methylation, DNA hydroxymethylation, histone methylation).

We do not expect technical difficulties in conducting the experiments as we already have experience in studying ELS effects on brain development and behaviour of transgenic models.

All equipment and housing needed for experiments are available at the institute to which our research group belongs.

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?

This project addresses the influence of an environmental factor interacting with a genetic factor in the development of stress-related disorders. It also provides mechanistic insights in the effects of *cumulative* ELS on the development of stress-related disorders, especially in relation to the serotonin transporter genotype. Good animal models are indispensable in this research, as we can invasively study the underlying mechanisms in the brain of animals exposed to ELS mediated by the maternal stress (pregestational and prenatal), something that is not possible in humans.

If the outcome shows that more rats express a depressive-like phenotype as a result of *cumulative* ELS, we will be able to elucidate the underlying mechanisms contributing to stress-induced disorders. In addition, if $SERT^{+/-}$ rats exhibit higher vulnerability to developing a depressive-like phenotype following the *cumulative* ELS exposure, we will be able to demonstrate that the serotonin transporter genotype moderates the influence of stress on depression. This would greatly benefit the clinical management of stress-induced disorders as new targets for drug treatment may be revealed.

Additionally, if altered social behaviours are observed in adult and especially also in young animals exposed to *cumulative* ELS, we will be able to show a different neurodevelopmental trajectory of social functioning induced by *cumulative* ELS presumably related to the late onset of depression.

Lastly, by studying if the parental depressive-like phenotype induced by *cumulative* ELS is transferred to the next generation, we will provide evidence of epigenetic signatures of the depressive-like phenotype shared between parents and the offspring.

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

Our research group along with other researchers conducting basic research on stress-related disorders, including depression, can use this animal model to further investigate neurobiological underpinnings related to how *cumulative* ELS influences the development of the brain to induce depressive-like behaviours, both separately and in combination with the SERT genotype. This model would also allow further investigation of the mechanisms underlying transgenerational effects of depression and the onset of social dysfunctions related to depression.

In the long-term, patients with depression might benefit from our studies when it will reveal new treatment targets.

Also in the long term, pharmaceutical companies focused on developing pharmacological agents may be interested in this animal model to test the efficacy of different drugs in treating depressive-related symptoms.

As experimental subjects, the condition of rats as stakeholders is done under the principles of the 3Rs and setting go/no-go moments. The refinement of depression models (cumulative ELS and/or SERT genotype) can lead to a decrease in the number of animals used for this purpose in the future.

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.

Phase 1: Pilot study

We will expose wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ rats to *cumulative* ELS to test whether they develop depressive-like behaviours when they are adults.

For this research project *cumulative* ELS refers to the combination of (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS exposure (see appendix 1 to this form for further description of each ELS type). $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to this *cumulative* ELS will be compared with $SERT^{+/+}$ and $SERT^{+/-}$ control animals.

We will test affective-related behaviour, cognitive performance and social functioning to obtain a comprehensive behavioral phenotype of the depressive-like rat model.

Milestone #1: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses of rats exposed to *cumulative* ELS compared to rats not exposed to ELS.
Milestone #2: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses induced by *cumulative* ELS are significant more impaired in $SERT^{+/-}$ rats compared to $SERT^{+/+}$ rats.

Selection points and decision criteria

The design and execution of phase 2 will be based on how the questions in the black squares below are solved according to findings in the pilot study:

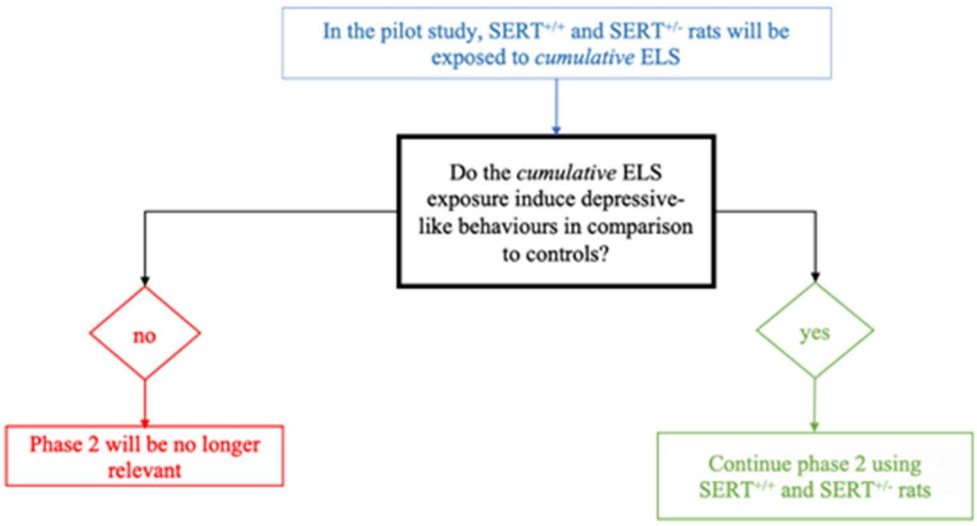

Fig1. Decision criteria to conduct phase 2 based on findings of phase 1. ELS: Early life stress; SERT: Serotonin Transporter.

If depressive-like behaviours are not expressed in animals exposed to *cumulative* ELS in comparison to controls in the pilot study, we will not continue to phase 2. If depressive-like behaviours are induced by cumulative stress, we will continue with phase 2 with both $SERT^{+/-}$ and $SERT^{+/+}$ rats.

Phase 2

Study 1: the depressive-like phenotype by *cumulative* ELS

We will expose $SERT^{+/+}$ and $SERT^{+/-}$ rats to *cumulative* ELS, to investigate whether they develop depressive-like behaviours when they are adults after being exposed to different combinations of ELS. Four conditions, one control and three experimental, will be compared in this study:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition 1: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to postnatal ELS (ELS treatment 1).
Experimental condition 2: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) prenatal ELS and postnatal ELS (ELS treatment 2).
Experimental condition 3 (same as pilot study): $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS (ELS treatment 3).

We will test affective-related behaviour, cognitive performance and social functioning in adult rats. We will also analyse mRNA expression level of selected genes, and epigenetic markers of those genes, in brain regions that are known to be involved in depression. Selected genes will at least include those involved in the serotonergic system, brain stress system (HPA axis), myelination and neural growth and survival as many of them can be co-expressed in major neural circuit connections involved in depressive-like behaviours in rodents [34]. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures and molecular analyses.

Milestone #1: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses of rats exposed to *cumulative* ELS compared to rats not exposed to ELS
Milestone #2: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses induced by ELS are significant more impaired in $SERT^{+/-}$ rats compared to $SERT^{+/+}$ rats.
Milestone #3: altered gene expression in brain tissue of rats exposed to experimental stress conditions compared to controls.
Milestone #4: altered DNA methylation (correlated to gene expression) at the promotor regions of selected genes in brain tissue of rats exposed to experimental stress conditions compared to controls.

Selection points and decision criteria

The design and execution of studies 2 to 5 will be based on how the questions in the black squares below are solved according to findings in study 1:

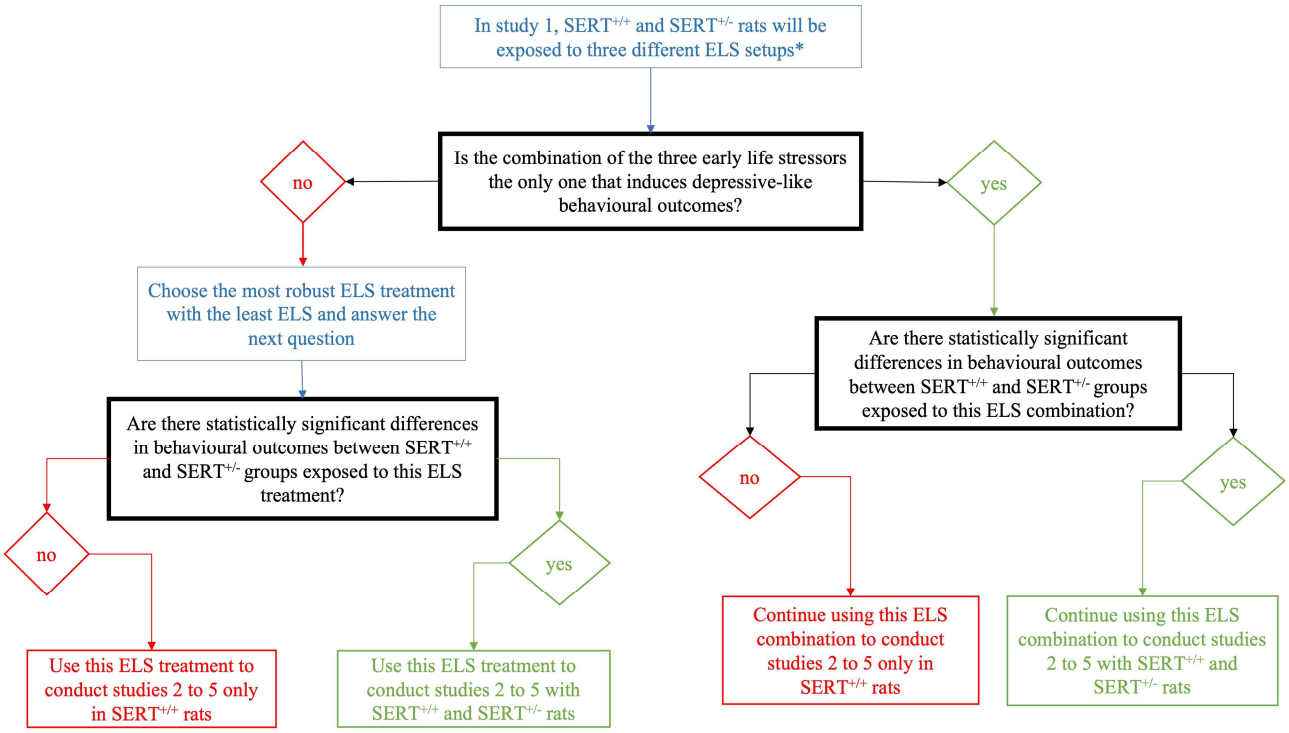

Fig2. Decision criteria to conduct studies 2 to 5 based on findings of study 1. ELS: Early life stress; SERT: Serotonin Transporter. *The three ELS treatments refer to the experimental conditions 1, 2 and 3 indicated in the text above (page 7).

Based on study 1, we will determine if the *pregestational, prenatal and postnatal* ELS is the most robust treatment to induce a depressive-like behavioral phenotype over the other two ELS treatments, and we will use it to the following studies. If not, we will select the ELS treatment that most robustly induces the depressive-like phenotype, with the least stressors, to use in the following studies and will discard the others. In addition, if $SERT^{+/-}$ rats do not express more vulnerability to depressive-like behaviours than $SERT^{+/+}$ rats, then $SERT^{+/-}$ rats will no longer be used in studies 2 to 5, and only $SERT^{+/+}$ animals will be included.

Study 2: focus on offspring social functioning in the juvenile period

We will expose $SERT^{+/+}$ and (depending on study1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. Next, we will compare social play behaviour between them when they are juveniles (both males and females). After the social play test, we will collect the brain tissue to analyse neuronal activity – through c-Fos expression by immunohistochemistry- in brain regions that regulate social play behaviour (e.g., prefrontal cortex, dorsal and ventral striatum, amygdala, habenula; [35]). See appendix 1 to this form for detailed description of the general design of the animal procedures.

Milestone #1: reduced social play behaviour and altered c-Fos expression in prefrontal cortex, dorsal and ventral striatum, amygdala, or habenula, all brain regions that regulate this behaviour between animals exposed to the ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Study 3: focus on offspring social functioning in adulthood

We will expose $SERT^{+/+}$ and (depending on study1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. When animals are adults, we will assess sexual behaviour and aggressive behaviour in both males and females. After the last aggression test, we will collect the brain tissue to analyse neuronal activity -through c-Fos expression by immunohistochemistry- in regions of the social brain network that regulate this behaviour (e.g., medial preoptical area, periaqueductal grey

matter, ventromedial hypothalamus, medial amygdala, [36]). See appendix 1 to this form for detailed description of the general design of the animal procedures.

Encountering intruders in their own territory induce a stress response in male and female rodents [37]. One way to measure this response is by assessing corticosterone (the main stress hormone in rodents) after the rat encounters an intruder in his/her territory [37,38]. Hence, when we test aggressive behaviour, we will take the aggression test as an opportunity to evaluate if corticosterone levels are differently affected by ELS treatment, SERT genotype, or its combination when the rat encounters an intruder in his/her own home cage.

Milestone #1: decreased sexual and aggressive behaviours between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).
Milestone #2: altered c-Fos expression after the aggression test, in brain areas that regulate aggressive behaviour between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).
Milestone #3: altered corticosterone levels following the aggression test between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Note: detailed procedures to test social play, sexual and aggressive behaviours and c-Fos expression in brain regions related to these behaviours are described in the appendix 1 of this proposal, section A, studies 2 and 3, respectively. Supported literature is provided correspondingly.

Study 4: unravelling molecular mechanisms of social functioning
We will evaluate the mRNA expression for selected genes, and epigenetic markers of those genes, in brain regions of the social brain network [35]. We will expose $SERT^{+/+}$ and (depending on study 1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. Next, animals will be left undisturbed until they are ±24 weeks old, when we will collect their brain tissue. This time of brain tissue collection is selected to be similar to the time in which we will collect the brain tissue in study 3. Selected genes will at least include those that code for vasopressin, serotonin, corticoid and oxytocin receptors as many of them can be co-expressed in several regions of the social brain network. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures.

Milestone #1: Altered gene expression in brain areas important for social behaviour in rats exposed to ELS compared to controls.
Milestone #2: altered DNA methylation at the promotor regions of selected genes in brain regions selected for social behaviour in rats exposed to ELS compared to controls.
Milestone #3: significant different gene expression/ DNA methylation in $SERT^{+/-}$ rats compared to $SERT^{+/+}$ rats after ELS.

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
We will determine if the depressive-like phenotype in one generation is transferable to their offspring. To accomplish this, first we will induce a depressive-like phenotype by exposing $SERT^{+/+}$ and $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. ($SERT^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, only $SERT^{+/+}$ rats will be used).

When F1 are adults, we will test their affective-related behaviour to confirm that the depressive-like phenotype is expressed. Then, depressed-like F1 males and depressed-like F1 females will be mated with wildtype, control females and males, respectively. The offspring of F1 will be referred as F2.

After delivery of F2, social communication of F2 pups and maternal care of F1 females during the first postnatal week of F2 will be tested. See appendix 1 to this form, section A for further explanation of these outcomes as indicatives of an altered behavioural phenotype.

To test whether the depressive-like phenotype is expressed in F2, affective-related, cognitive, and social behaviours, gene expression and epigenetic markers of F2 will be assessed. Selected genes for mRNA expression analysis and measurement of epigenetic markers will be the same as for the study 1. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures.

Milestone #1: Increased affective-related, or decreased cognitive or social behaviours between F2 born from depressed-like parents compared to F2 born from non-depressed-like parents.
Milestone #2: disturbed maternal care behaviour of depressed-like F1 female rats compared to non-depressed-like F1 female rats.
Milestone #3: Altered gene expression of selected genes of F2 born from depressed-like parents compared to F2 born from non-depressed-like parents.
Milestone #4: altered DNA methylation at the promotor regions of selected genes of F2 born from depressed-like parents compared to F2 born from non-depressed-like parents.

Milestone #5: similar DNA methylation profile at promotor regions of selected genes between depressed-like F1 and depressed-like F2.

Coherence: From a pilot study, we will investigate whether the combination of pregestational, prenatal and postnatal cumulative ELS induces a depressive-like behavioural phenotype (Phase 1). Next, we will explore whether this phenotype can also be reached with less early life stressors (Phase 2: study 1), and determine whether $SERT^{+/-}$ rats are more vulnerable than wildtypes (Phase 2: study 1). Also, we will explore the underlying molecular mechanisms of this depressive-like rat model (Phase 2: study 1). Follow-up studies will then zoom in on the social functioning in depression, its neurobiological mechanisms, and transgenerational effects (phase 2: studies 2 to 5).

3.4.2 Provide a justification for the strategy described above.

All studies proposed above are based on the principles of experimental research. For each study we have:

1. Established hypothesis to be empirically tested
2. Defined independent variables to be manipulated
3. Defined dependent variables to be measured
4. Followed experimental designs to establish relevant groups of comparison and control unknown and/or confounding variables

By following this strategy, we seek to establish a causal relationship between variables. We expect to demonstrate that the occurrence of depressive-like behaviours, altered gene expression and distinctive epigenetic markers of relevant genes (dependent variables) arise due to *cumulative* ELS exposure alone or in combination with the SERT genotype (independent variables).

The order in which the studies will be performed is important. In study 1 we will test if *cumulative* ELS induces a depressive-like phenotype in a robust fashion, alters gene expression and changes epigenetic signatures. We will only perform the following studies if a significant effect is found in this study. In studies 2 to 4 we will further characterise the social functioning and neurobiological mechanisms involved. Finally, we will investigate if the offspring (F2) of depressive-like parents (F1) also express a depressive-like phenotype, exhibit altered mRNA expression, and manifest changes in epigenetic markers.

By conducting these studies, we will answer the question whether the interaction of *cumulative* ELS and the SERT genotype increases the vulnerability to develop a depressive-like phenotype and provide mechanistic insights in stress-related disorders. In addition, we will understand how the neurodevelopmental trajectory of social functioning in depression, induced by ELS, is characterised. Finally, we will know to what extent the parental depressive-like phenotype is transferred to the next generation.

The selected strategy will allow us to optimise the use of animals and reduce their number where possible. The study 1 will be critical in this respect. Only the ELS treatment that produces the most robust depressive-like phenotype will be used in the follow-up studies. In addition, if no clear SERT genotype effect is found, the SERT$^{+/-}$ animal will not be used in the following studies.

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	Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.

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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 (0800-7890789).

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: Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter

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.

Phase 1
Pilot study
We will expose wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ rats to *cumulative* ELS to test whether they develop depressive-like behaviours when they are adults.

For this research project *cumulative* ELS refers to the combination of (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS exposure. See the next section for detailed procedure of each type of ELS. $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to this *cumulative* ELS will be compared with $SERT^{+/+}$ and $SERT^{+/-}$ control animals.

From 14 weeks of life, $SERT^{+/+}$ and $SERT^{+/-}$ rats will be assessed for affective-related, cognitive and social behaviours. For this assessment, we will use the behavioural tests indicated below:

1- Elevated plus maze (EPM), sucrose preference test (SPT), and open field test (OFT) to evaluate affective-related behaviours.
2- Object location test (OLT) and novel object recognition (NOR) to test cognitive performance.
3- Social interaction test and social recognition test to assess social investigation, social memory and social withdrawal.

The tests will be conducted at least one week apart in the following order: EPM, SPT, OFT, OLT, NOR, social interaction test, and social recognition test. See next section about description of proposed animal procedures for detailed procedure of each behavioural test. After the last behavioural test (±20 weeks), animals will be sacrificed by fast decapitation and their brain tissue will be collected.

The chronology of this pilot study is summarised in fig1:

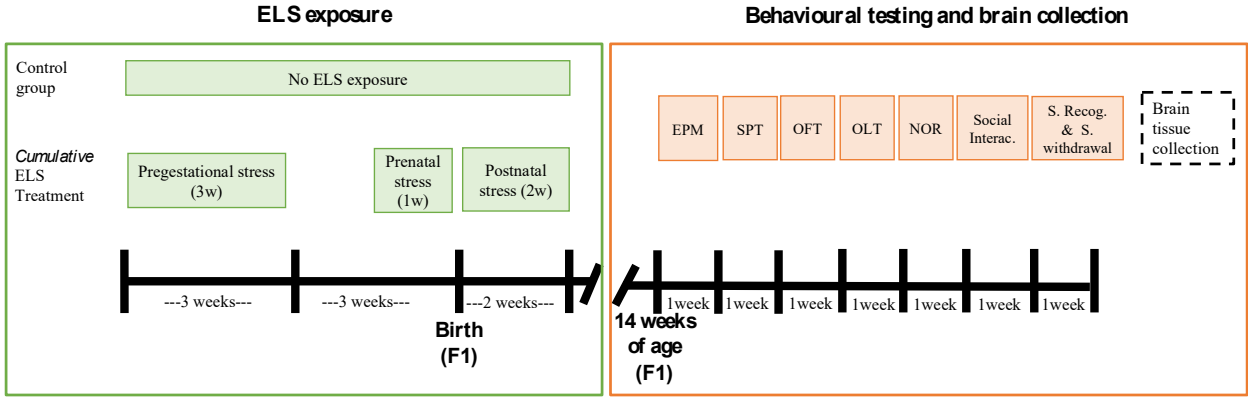

Fig1. General design of the pilot study.

-The EPM is useful test to evaluate anxiety-related behaviours. It is based on the natural tendency of rodents to avoid open spaces and stay in enclosed spaces. The more exploration in open arms, the more indication of reduced levels of anxiety.
-The SPT aims to evaluate the capacity of an animal to experience pleasure by consuming palatable food. It is based on the natural preference of rodents for sweets. Low levels of sucrose consumption are indicative of anhedonia, a central symptom in depressive disorders
-The OFT aims to evaluate anxiety-like behaviours. It is based on the natural tendency of rodents to avoid open spaces and exhibit thigmotaxis. The more time the animal spends walking along the walls or staying in the corners of the arena, the higher the index of anxiety-like responses.
-The OLT is useful to evaluate spatial short-term and long-term memory in rats. It is based on the spontaneous tendency of rodents, previously exposed to two identical objects, to later explore one of the objects—placed in a novel location—for a longer time than they explore the non-displaced object. The more time the animal spends with the object in the new location, the higher the level of spatial memory.
-The NOR aims to evaluate short and long-term recognition memory. It is based on rodents' natural tendency to explore novel features in their environment, including objects. The more time the animal spends with the new object, the higher the level of recognition
-Social interaction test, performed in an open arena, aims to evaluate investigation towards new social stimuli. It is based on the natural tendency of rodents to investigate unfamiliar mates. Several behaviours such as sniffing, grooming, or mounting can be measured as an index of social interaction.
-Social recognition test aims to assess the ability to recognise a novel mate in comparison to familiar mates. This test is based on the natural tendency of rodents to investigate unfamiliar partners. By performing this test in a three-chamber box, the more time spent in the box with the novel partner, the higher the index of social discrimination. An additional benefit of using this apparatus is the possibility to evaluate social withdrawal, i.e., the lack of motivation to have social contact. Social withdrawal can be assessed by measuring the time spent in the box in which neither the familiar nor the unfamiliar mate are present.

Two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to *cumulative* ELS.

This pilot study will follow a factorial experimental design with two factors: Factor 1: ELS treatment; Factor 2: SERT genotype.

The primary outcome parameters to test the onset of depressive-like behaviours induced by *cumulative* ELS will be the responses displayed by the rats in the behavioural tests mentioned above (also shown in orange boxes of fig1). The selection of these tests is based on previous research conducted in our research group and elsewhere [2-4] showing their validity to assess several parameters of affective-related behaviour, cognition, and social behaviours in rodents to obtain a comprehensive behavioral phenotype of the depressive-like rat model.

Phase 2

Study 1: the depressive-like phenotype by *cumulative* early life stress (ELS)

We will expose SERT$^{+/+}$ and SERT$^{+/-}$ rats to *cumulative* ELS to confirm whether they develop depressive-like behaviours in a robust fashion when they are adults. In contrast with the pilot study however, four conditions, one control and three experimental, will be compared in this study:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.
Experimental condition 1: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to postnatal ELS (ELS treatment 1).
Experimental condition 2: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to (maternal) prenatal ELS and postnatal ELS (ELS treatment 2).
Experimental condition 3 (*cumulative* ELS) : SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS (ELS treatment 3).

Similar to the pilot study, at 14 weeks of life rats will start to be behaviourally tested. See description of the pilot study above for further details about each behavioural paradigm and the order in which they will be used. After the last behavioural test (±20 weeks), animals will be sacrificed by fast decapitation and their brain tissue will be collected to analyse gene expression of selected genes, and epigenetic markers of those genes, in brain regions that are known to be involved in depression. Selected genes will at least include those involved in the serotonergic system, brain stress system (HPA axis), myelination, neural growth and survival as many of them can be co-expressed in major neural circuit connections involved in depressive-like behaviours in the rat (e.g., prefrontal cortex, hippocampus, amygdala) [1].

The chronology of this study is summarised in fig2:

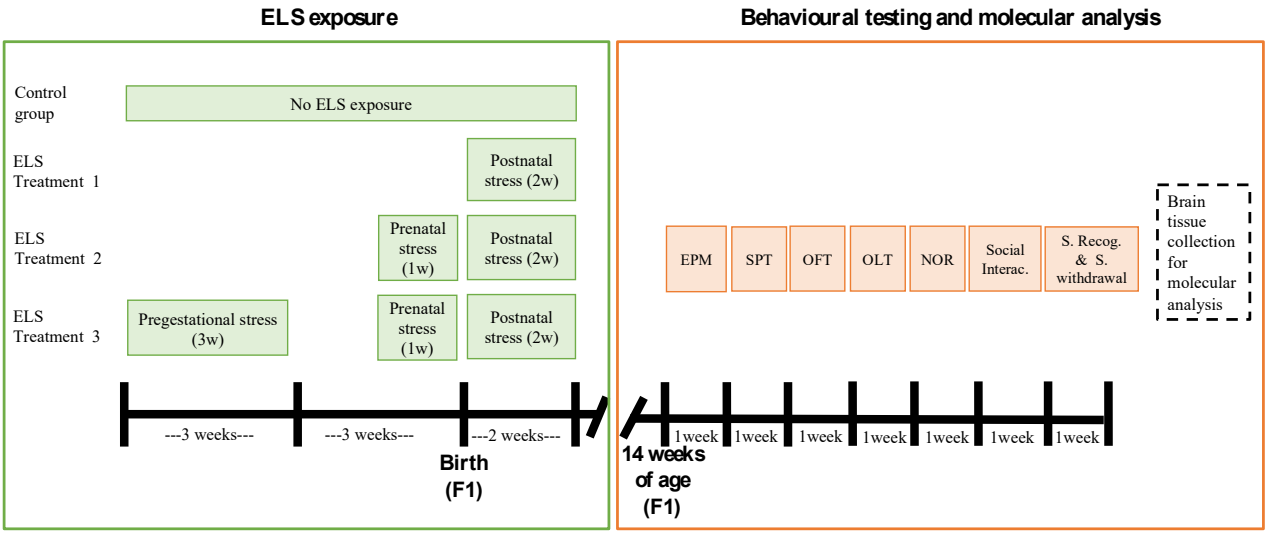

Fig2. General design of the study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: SERT genotype; Factor 3: Sex.

The primary outcome parameters to test the onset of depressive-like behaviours induced by *cumulative* ELS will be the responses displayed by the rats in the behavioural tests mentioned above (also shown in orange boxes of fig2). The selection of these tests is based on previous research conducted in our research group and elsewhere [2-4] showing their validity to assess several parameters of affective-related behaviour, cognition, and social behaviours in rodents.

The primary outcome parameter to test the changes of gene expression in the brain regions related to depressive-like behaviours induced by *cumulative* ELS will be the level of mRNA expressed for selected genes. The selection of mRNA gene expression analysis is based on previous evidence indicating that lower levels of mRNA expression of genes related to serotonergic system, brain stress system and neural growth correlated with maladaptive responses that increases the risk for depression [5]. Quantification of mRNA expression will be done by using the quantitative polymerase chain reaction (RT-qPCR) technique.

The primary outcome parameter to test epigenetic markers induced by *cumulative* ELS will be the level of DNA methylation at the promotor regions of selected genes. The DNA methylation analysis is selected because changes in DNA methylation (in general, hypermethylation) have been associated with depression [6].

Measurement of DNA methylation status in promotor regions of selected genes will be performed by PCR amplification of bisulfite-converted DNA [7].

It should be noted that additional ELS treatments without postnatal ELS are not relevant to this research project. If we exclude this period, the model will not resemble an important aspect of the human condition we seek to translate into the animal, i.e., adversity during the first years of life as a risk factor for depression later in life.

Studies 2 to 5 will be performed *only* if a depressive-like behavioural phenotype is found in this study. See section 3.4.1 of the project proposal form to see the explanation of selection points and decision criteria in this respect.

Study 2: focus on offspring social functioning in the juvenile period
Here we will test whether *cumulative* ELS alters social functioning in the juvenile period, and whether $SERT^{+/-}$ rats are more vulnerable compared with $SERT^{+/+}$ rats (depending on study1). To accomplish this, $SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

At ±4-5 weeks of age, animals will be assessed for social play behaviour in the social play-fighting test. This test will take place for 20 minutes (see next section for detailed procedure of social play-fighting test). After 90-120 minutes the social play-fighting test is over, animals will be sacrificed and brain tissue will be collected by perfusion to analyse the c-Fos protein expression level in brain regions that regulate social play behaviour (e.g., prefrontal cortex, dorsal and ventral striatum, amygdala, habenula) [8].

The chronology of this study is summarised in fig3:

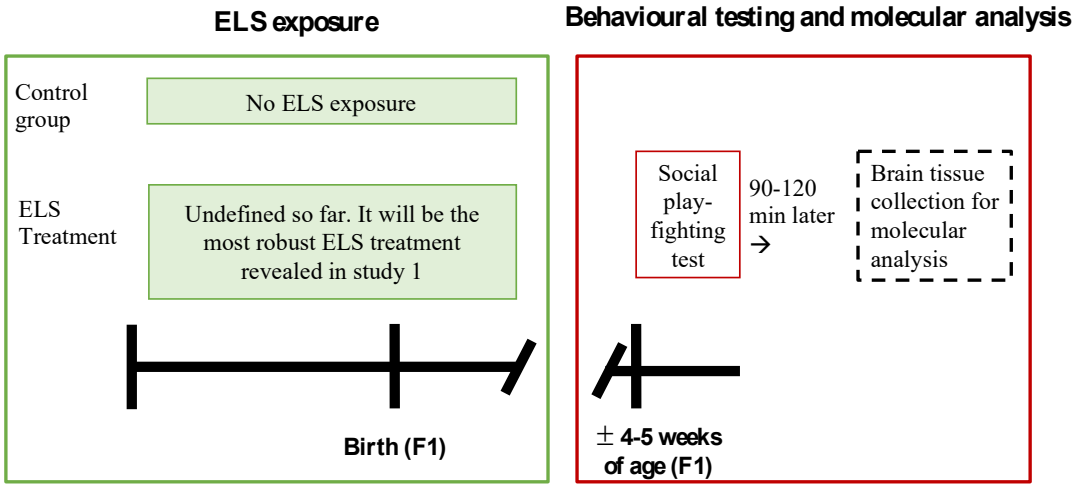

Fig3. General design of study 2

Two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if $SERT^{+/-}$ animals are included).

The primary outcome parameters to test the changes in social play behaviour induced by *cumulative* ELS will be the responses displayed by the rats in the social play-fighting test. This test is selected because it allows to assess behavioural patterns of social play behaviour that are highly expressed in rodents at 35-42 days of age [9].

The primary outcome parameter to test changes in neuronal activity of brain regions that regulate social play behaviour induced by *cumulative* ELS will be the level of c-Fos protein expression in neurons of such brain regions. This expression level will be quantified through c-FOS protein immunohistochemistry staining to count c-Fos-positive neurons. c-Fos protein is expressed rapidly and transiently after external stimuli [10].

Therefore, the c-Fos protein expression level will serve as an indirect biomarker of neuronal activity in brain areas that regulate social play behaviour during the social play-fighting test.

Study 3: focus on offspring social functioning in adulthood
Here we will test whether *cumulative* ELS alters social functioning in adulthood, and whether SERT$^{+/-}$ rats are more vulnerable (depending on study1). To accomplish this, SERT$^{+/+}$ and SERT$^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

From 14 weeks of age, sexual behaviour of males and females will be tested once a week for 6-10 consecutive weeks. In order to prevent pregnancy in females during the sexual behaviour testing, a double tubal ligation surgery will be performed two weeks before the behavioural testing begins. See next section about description of proposed animal procedures of study 3, for details of this surgery procedure.

To test sexual behaviour in males, we will place the rat with a stimulus female in a testing cage. To test sexual behaviour in females, we will place the rat with a stimulus male in a testing cage. After the last sexual test has been taken place, males will be housed individually with a companion female in a new home cage to elicit territorial behaviour. Likewise, females will be housed individually in a new home cage to elicit territorial behaviour. One week later, we will use the resident-intruder test in males, and the female-intruder test in females to test aggressive behaviour. See next section about description of proposed animal procedures of study 3, for detailed procedure of each behavioural test.

As aggressive encounters induce a stress response in animals, we evaluate the stress response induced by the resident-intruder test in males and the female-intruder test in females. We will collect three blood samples from the animals' tail to assess the concentration level of corticosterone (the main stress hormone in rodents) in systemic circulation. We will collect three blood samples in total (one before, and two after the aggression test) because this will help us to track the changes in the corticosterone level as a result of being exposed to a stress challenge (i.e., the aggression test). See next section about description of proposed animal procedures of study 3, for detailed procedure of blood sampling.

After the resident-intruder test and collection of blood samples (90-120 minutes), animals will be sacrificed, and the brain tissue will be collected by perfusion to analyse the c-Fos expression level in brain regions that regulate aggressive behaviour [11].

The chronology of this study is summarised in fig4:

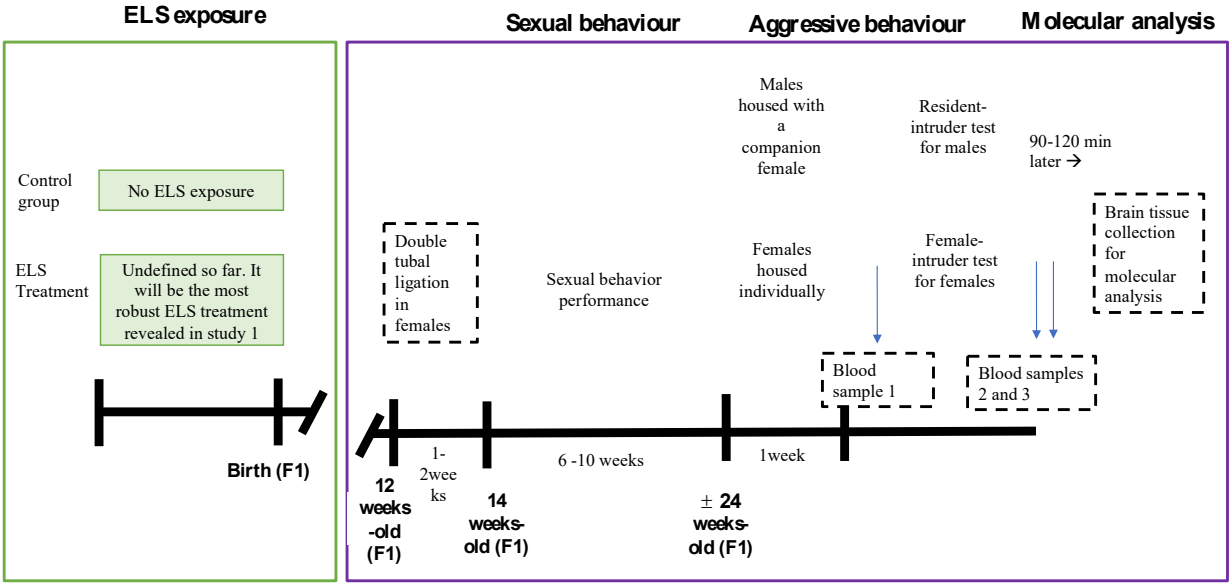

Fig4. General design of study 3. The symbol ± means approximately. It is possible that sexual behaviour testing takes fewer than 10 weeks. Therefore, the individual housing of males and females, the aggression test and the brain collection may take place few weeks earlier.

Two conditions, one control and one experimental, will be compared:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.

Experimental condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if SERT$^{+/-}$ animals are included).

Readouts of sexual behaviour test, resident-intruder test, and female-intruder test will be the primary outcome parameters to test the changes in adult social behaviour induced by *cumulative* ELS. Sexual behaviour testing is selected because adult males express copulatory behaviours in the presence of females, and females express proceptive and receptive behaviours (mediated by the oestrus cycle) in the presence of males. The resident-intruder test for males and the female-intruder test for females are selected because adult rodents express territorial aggression when encountering intruders in their own territory.

Systemic concentration level of corticosterone will be the primary outcome parameter to test whether *cumulative* ELS alters the stress response in adult rats following the exposure to a stress challenge (i.e., the aggression test). Blood sampling is selected because corticosterone is released from adrenal glands into blood circulation.

c-Fos-positive neurons expressed in brain regions that regulate aggressive behaviour will be the primary outcome parameter to test changes in neuronal activity induced by *cumulative* ELS. As mentioned above, c-Fos protein is expressed rapidly and transiently after external stimuli [10]. Therefore, the c-Fos protein expression level will serve as an indirect biomarker of neuronal activity in brain areas that regulate the aggressive behaviour during the aggression test.

Study 4: unravelling molecular mechanisms of social functioning
Here we will test the underlying molecular mechanisms related to the social brain network in adult animals exposed to *cumulative* ELS, and whether SERT$^{+/-}$ rats exhibit a different molecular pattern in comparison to SERT$^{+/+}$ (depending on study 1). To accomplish this, SERT$^{+/+}$ and SERT$^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

The animals will be left undisturbed (except for cage cleaning) until ±24 week of age in which they will be sacrificed by decapitation to collect the brain tissue. We will analyse the mRNA expression of selected genes in brain regions of the social brain network. We will also test the DNA methylation level at the promotors of those selected genes. Selected genes will at least include those that code for vasopressin, serotonin, corticoid and oxytocin receptors as many of them can be co-expressed in several regions of the social brain network.

The chronology of this study is summarised in fig5:

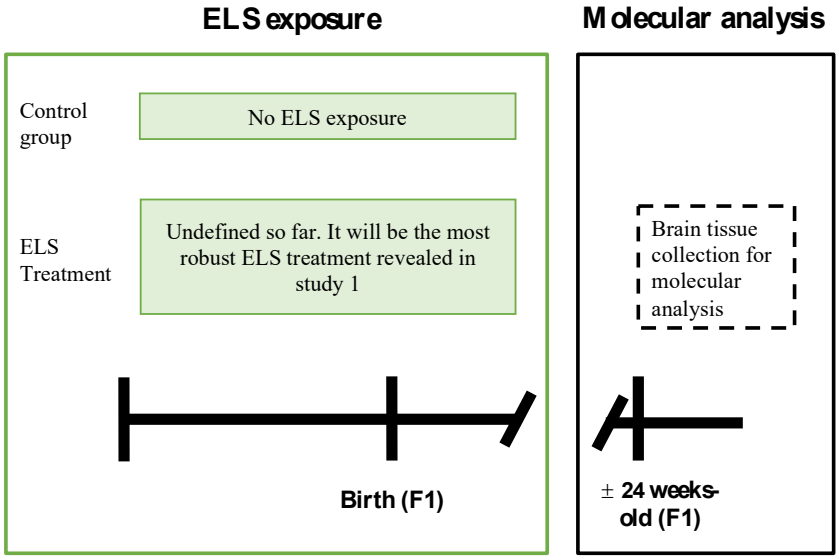

Fig5. General design of study 4. The symbol ± means approximately. This time point may vary depending on the time in which brain tissue will be collected in study 3.

Two conditions, one control and one experimental, will be compared:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.

Experimental condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if SERT$^{+/-}$ animals are included).

No behavioural procedures will be performed before brain collection to prevent changes in gene expression and epigenetic markers as a result of behavioural tests. We will collect brains at ±24 week of age to make these results comparable to findings in study 3.

The primary outcome parameter to test the changes of gene expression in brain regions of the social brain network induced by *cumulative* ELS will be the level of mRNA expressed for selected genes. The selection of mRNA gene expression analysis is based on previous evidence indicating that gene expression of selected genes in brain regions comprising the social brain network are closely linked to expression of social behaviours like aggression [12]. Quantification of mRNA expression will be done by using the quantitative polymerase chain reaction (RT-qPCR) technique.

The primary outcome parameter to test epigenetic markers induced by *cumulative* ELS will be the level of DNA methylation at the promotor regions of selected genes in brain regions of the social brain network. The DNA methylation analysis is selected because changes in DNA methylation (in general, hypermethylation) have been associated with vulnerability to abnormal social functioning [13]. Measurement of DNA methylation status in promotor regions of selected genes will be performed by PCR amplification of bisulfite-converted DNA [7].

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
Here, we will determine if the depressive-like phenotype in generation F1 is transferable to their offspring F2. To accomplish this, first we will induce a depressive-like phenotype in F1 by exposing SERT$^{+/+}$ and SERT$^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. (SERT$^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, only SERT$^{+/+}$ rats exposed to ELS will be used).

When F1 males and females are adults, we will test affective-related behaviours to confirm that the depressive-like phenotype is expressed. To accomplish this, from 14 weeks of age (similar to study 1), affective-like behaviours of F1 will be tested by using the same tests used in study 1 (i.e., EPM, SPT and OFT). The tests will be conducted at least one week apart in the following order: EPM, SPT, OFT. After the last behavioural test, F1 animals will be mated to create F2. To do so, depressed-like males and depressed-like females will be mated with wildtype, control females and males, respectively.

F1 pregnant females will be left undisturbed during pregnancy (except for cage cleaning). After delivery of F2, we will measure social communication of F2 pups on postnatal day 6 and maternal care of F1 females during the first postnatal week of F2. See next section about description of proposed animal procedures of study 5, for detailed procedure of these behavioural tests.

When F2 are 14 weeks old (similar to study 1), we will test if the depressive-like phenotype is expressed. To do so, we will test affective-related, cognitive, and social behaviours of F2 as well as molecular mechanisms involved, by proceeding the same as we will proceed in study 1. Therefore, the same order and type of behavioural tests, as well as mRNA expression analysis and DNA methylation analysis will be conducted.

The chronology of this study is summarised in fig6:

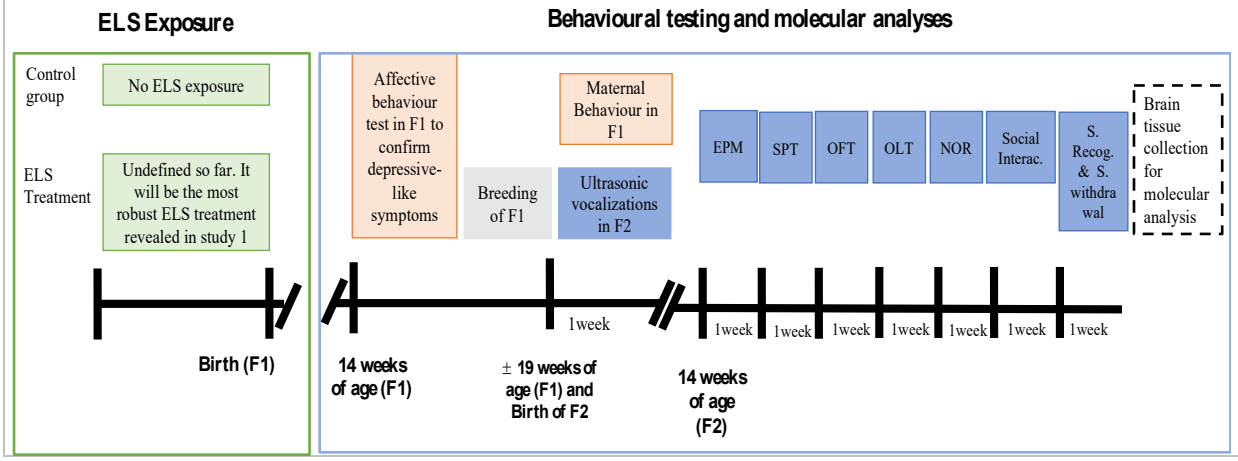

Fig6. General design of study 5. The symbol ± means approximately. Successful breeding of F1 may take longer. It is possible that depressed-like females will not get pregnant in the first attempt of breeding.

F1 and F2 outcomes will be analysed separately.

F1 analysis:
To analyse affective-related behaviours and maternal care of F1, two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

The primary outcome parameter to confirm the depressive-like phenotype in F1 will be the responses that they will display in EPM, SPT and OFT.

The primary outcome parameter to test altered maternal care in F1 females will be the responses of care towards the pups expressed by the dam in the nest. Altered maternal care in humans is a clinical feature of postpartum depression (a type of depression); therefore, we will test whether the depressive-like phenotype in F1 females alters the maternal care.

F2 analysis:
To analyse altered social communication, depressive-like phenotype, gene expression, and epigenetic marks of F2, four conditions, one control and three experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats born from $SERT^{+/-}$ F1 males mated with control $SERT^{+/+}$ F1 females.

Experimental condition 1: $SERT^{+/+}$ rats born from early-life stressed $SERT^{+/+}$ F1 males mated with control $SERT^{+/+}$ F1 females.

Experimental condition 2: $SERT^{+/+}$ rats born from early-life stressed $SERT^{+/+}$ F1 females mated with control $SERT^{+/+}$ F1 males.

Experimental condition 3: $SERT^{+/+}$ and $SERT^{+/-}$ rats born from early-life stressed $SERT^{+/-}$ F1 males mated with control $SERT^{+/+}$ F1 females.

Experimental condition 4: $SERT^{+/+}$ and $SERT^{+/-}$ rats born from early-life stressed $SERT^{+/-}$ F1 females mated with control $SERT^{+/+}$ F1 males.

The primary outcome parameter to test altered social communication in F2 pups induced by the F1 depressive-like phenotype will be the ultrasonic vocalizations emitted by the pups. The selection of this test is based on the natural ability of pups to transmit affective-related states to their mother through ultrasonic vocalizations. Reduced ultrasonic vocalizations of F2 will be an indicator of dysfunctional social communication very early in life as a result of being born from depressed-like parents.

The primary outcome parameters to test the F2 depressive-like phenotype induced by F1 parental-like depression will be the responses displayed by F2 rats in the behavioural tests indicated above (also shown in the blue boxes of fig6).

The primary outcome parameters to test changes in gene expression and epigenetic markers of F2 induced by F1 parental-like depression will be the level of mRNA expressed of selected genes and the level of DNA methylation at the promotor regions of selected genes in brain regions that are known to be involved in depression.

The selection of these behavioural tests and molecular analyses is based on evidence of offspring neurobiological and behavioural outcomes associated with stress/depression in the mother [14-16] and the father [17,18]. It is suggested that not only maternal stress can induce epigenetically driven effects on the offspring, but also paternal stress can induce long-lasting changes in germ cells, thus potentially inducing changes across generations [17].

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

Phase 1
Pilot study
SERT$^{+/+}$ and SERT$^{+/-}$ rats will be exposed to *cumulative* ELS. The procedures to induce (maternal) pregestational ELS, (maternal) prenatal ELS, and postnatal ELS are described below:

1) *pregestational ELS* refers to stressful events experienced by females before pregnancy to interfere with the development of the offspring. Pregestational stress will be applied to females for at least 21 days before pregnancy, induced by chronic unpredictable stress. We will follow the same protocol as [19,20]. Based on this protocol, females will be housed individually and subjected to 1–2 stressors per day for 3 weeks. Stressors will include restraint under bright light (1000 lux) for 1 h, overcrowding overnight (4 females per Macrolon type III cage), overnight exposure to damp bedding, 12h of food restriction, 5 minutes of forced swimming, and cage rotation for 12h. In accordance with the *Nationaal Comité advies dierproevenbelid* advice to prevent withdrawal of food for more than 24 hours in animals used for neurocognitive research [21], 12 hours of food restriction will be the maximum time used in this procedure and the female will be exposed to environmental enrichment; in addition, food restriction will not be applied in two consecutive days. Cage rotation will consist of changing the home cage location from one place to another from morning to afternoon, within the same experimental room. We select chronic unpredictable stress due to its previous validation to induce sustained changes in the stress response and depression-like responses in rodents. This procedure has been effective to induce neural changes and behavioural impairments in the offspring when it is applied to females before pregnancy. It was shown that pregestational stress caused lower viability of pregnancy, so about 1/3 of females did not became pregnant [22]. However, we will do some rebreeding attempts (max 3 attempts) to cover that.

2) *prenatal ELS* refers to stressful events experienced by females during their pregnancy to interfere with the development of the offspring. We will follow the same protocol as van den Hove et al. 2006 [23]. Females will be exposed to stress during the last week of pregnancy (14-21days), subjected to 3 sessions of 45-min restraint stress each day while being exposed to bright light. Dams will be put inside a plastic tube in which they can move their paws but cannot turn around. Each 45-min stress session will be as unpredictable as possible. We select restraint stress due to its efficacy to induce changes in the stress response and increase the likelihood of expressing depresssive-like behaviours. This procedure has been effective in inducing neural changes as well as affective-related and social impairments in the offspring when it is applied to females in the last week of pregnancy. Based on the van den Hove et al procedure, prenatal stress had no effect on gestational length, litter size, or pre-weaning mortality [23].

3) *postnatal ELS* refers to stressful events experienced during the first weeks of life (in rodents). In this research project, SERT$^{+/+}$ and SERT$^{+/-}$ rats will be exposed during the first two weeks of life (PND 2-15) to unpredictable maternal separation and disrupted maternal care induced by maternal stress. We will follow the same protocol as [4]. Unpredictable maternal separation in combination with maternal stress produces more persistent behavioural effects in the offspring. As a consequence of disrupted maternal care, the offspring is at higher risk of developing affective-related impairments later in life.

To induce unpredictable maternal separation, pups will be transferred as a whole litter into a new room for 3h/per day, starting at unpredictable time points each day. The whole litter will be placed in preheated Makrolon type II cages to prevent hypothermia (postnatal days 1-8: 32±1 °C; postnatal days 9-15: 28±1 °C). To disrupt maternal care behaviours, mothers will be exposed to maternal stress. Maternal stress will consist of either 20-min restraint stress or 5-minute forced swimming in cold water. They will be applied unpredictably and randomly during the separation of the litter. For restraint stress, dams will be put inside a plastic tube in which they can move their paws but cannot turn around. For the forced swimming, females will be placed into a cylindrical Plexiglas tank filled up with water at 18 °C.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. From 14 weeks of life, SERT$^{+/+}$ and SERT$^{+/-}$ rats (2 males and 2 females maximum per litter) will be behaviourally tested. General procedure for each behavioural test is indicated below:

1) EPM: The rat will be taken from the home cage and placed in the centre of the maze facing an open arm. The rat will be allowed to freely explore the maze for 5 min. Afterwards, the animal will be returned to the home cage.
2) SPT: Within his/her home cage, the rat will be exposed to one bottle of water and one bottle containing a sucrose solution for 24h on alternating days. On the other days two bottles of water will be presented. With each sucrose day, the sucrose concentration will be increased. Sucrose bottle locations on the cage will be alternated on sucrose days to prevent spatial bias. This test will take place for one week in total.
3) OFT: The rat will be taken from the home cage and placed in the centre of the open arena to freely explore the open field for 10 minutes. Afterwards, the animal will be returned to the home cage.
4) OLT: The rat will be taken from the home cage and placed into an open arena with two identical objects placed in two opposite corners. Free exploration will be allowed for 3 min (trial 1). After that, both the rat and the objects will be removed for 1 hr (to test short-term memory), after of which the next trial will start (trial 2). In this trial, the animal will be exposed to the same two objects as trial 1 for another 3 min; however, this time one of the objects will be place in a novel location. After the trial 2 is over, the animal will be returned to the home cage.
5) NOR: The procedure will be the same as for OLT. In this case, trial 1 will consist of exposure to two identical objects whereas trial 2 will consist of exposure to one familiar and one novel object placed in the same location during both trials.
6) Social interaction test: After the habituation to the arena, two rats that are unfamiliar to each other will be placed simultaneously in an open arena for 10 minutes. Each couple of animals will be matched by sex, SERT genotype, and ELS treatment. Afterwards, both animals will be returned to the home cage.
7) Social recognition test. After 10-minute habituation, the experimental rat will be allowed to explore an unfamiliar younger stimulus rat (stranger 1) that will be placed under a plastic grid in the left or right chamber of a three-chamber box, while the other chamber will contain an empty grid. After 10-minute exploration, a second unfamiliar younger stimulus rat (stranger 2) will be placed in the empty grid, and 10-minute exploration will be allowed. Afterwards, the animal will be returned to the home cage.

After all behavioural tests have been taken place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored for further molecular analyses.

Phase 2

Study 1: the depressive-like phenotype by *cumulative* ELS

$SERT^{+/+}$ and $SERT^{+/-}$ rats will be exposed to one, two, or three types of ELS depending on the experimental conditions they will be allocated to. See the animal procedures to induce (maternal) pregestational ELS, (maternal) prenatal ELS, and postnatal ELS described in the pilot study above.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. From 14 weeks of life, $SERT^{+/+}$ and $SERT^{+/-}$ rats (2 males and 2 females maximum per litter) will be behaviourally tested. General procedure for each behavioural test is the same as the one described in the pilot study above.

After all behavioural tests have been taken place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Study 2: focus on social functioning in the juvenile period

$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. At ±4-5 week of age, animals will be assessed for social play behaviour in the social play-fighting test. For this test, animals will be tested in couples. Each couple will be considered as an experimental unit and will consist of two unfamiliar mates matched by sex, SERT genotype, and ELS treatment. After the habituation to the testing cage (5 minutes), animals will be tested for 15 minutes. Boxing/wrestling, pouncing, pinning, chasing and social grooming will be scored.

After 90-120 minutes the social play-fighting test is over, the rat will be taken to a different room and will be anesthetised. Then, the rat will be sacrificed by perfusion and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Study 3: focus on social functioning in adulthood
$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. As of 14 weeks of age, sexual behaviour of males and females will be tested once a week for 6-10 consecutive weeks. To test sexual behaviour in males, we will place the rat with a stimulus female in a testing cage for 30 minutes. To test sexual behaviour in females, we will place the rat with a stimulus male in a testing cage for 30 minutes. Table 1 below indicates the sexual responses that will be scored in males and females. Immediately after the test, rats will be returned to their home cage.

After the last sexual test has been taken place, males will be housed individually with a companion female in a new home cage to elicit territorial behaviour. Likewise, females will be housed individually in a new home cage to elicit territorial behaviour. Water and food will be provided *ad libitum*. For home cage enrichment, wooden gnawing sticks, a polycarbonate small box for resting/sleeping and autoclaved bedding material will be provided. One week later, aggressive behaviour will be tested. For this assessment, we will use the resident-intruder test in males, and the female-intruder test in females.

The resident-intruder test will consist of the introduction of an unfamiliar, intruder male into the home cage of the resident male. The female-intruder test will consist of the introduction of an unfamiliar, intruder female into the home cage of the resident female. In both cases, the resident will correspond to the experimental animal, while the intruder will correspond to a same-sex, smaller stimulus rat that is unknown for the experimental animal. The aggression test will take place for 30 minutes and the behaviour of the resident will be recorded. Table 1 below indicates the aggressive responses to be scored in males and females. Immediately after the aggression test is over, rats will be placed in their home cage. From 90 to 120 minutes after the aggression test has been taken place, the rat will be taken to a different room and will be anesthetised. Then, the rat will be sacrificed by perfusion and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Table 1. Readouts of sexual behaviour and aggressive behaviour

Sexual behaviour	**Aggression**
- Copulatory behaviour in males by scoring mounts, intromissions, and ejaculations - Receptive behaviour in females by scoring lordosis - Proceptive behaviour in females by scoring darts and hops and time spent with the male	- Attacks, lateral threat, upright posture, clinch attack, keep down and chase in males during the resident-intruder test. - Same parameters in females during the female-intruder test.

As aggressive encounters induce a stress response in animals, we will evaluate the stress response induced by the resident-intruder test in males and the female-intruder test in females. We will collect three blood samples from the animals' tail to assess the concentration level of corticosterone (the main stress hormone in rodents) in systemic circulation. Sampling will be made by making a small cut in the tail and collecting the blood from the dorsal tail vein. The first sample will be collected before the test to establish the baseline level of the hormone. The second sample will be collected immediately after the aggression test by removing the crust from the incision and collecting the blood. The third sample will be collected 30 minutes later by removing the crust (or making a new, small incision if needed) and collecting the blood. Each sampling will be maximum 300 uL, so the total amount of blood sampling will be < 1ml/kg. This procedure is considered as stress-free procedure and has been proven to be reliable to test corticosterone levels in systemic circulation in rodents [24].

Additional animal procedures in this study:
To prevent pregnancy in experimental females when the sexual behaviour is tested with the stimulus male, double tubal ligation surgery will be performed. This surgery will be also performed in stimulus females used to test sexual behaviour of experimental males. Likewise, same surgery will be performed in companion females

that will be caged with experimental males to conduct the resident-intruder test. In all three cases, the double tubal ligation surgery will be performed at least one week before the female is in contact with the male.

In preparation for the surgery, the rat will be anesthetised. To minimise discomfort directly linked to the surgery, analgesics will be subcutaneously injected at the start of the surgical procedure. The female will receive analgesics 24 hrs and 48 hrs hours after surgery. During the 7- 14-day recovery period, the weight and the well-being of the animals will be checked daily (on weekdays). Stimulus females used to test sexual behaviour of experimental males and companion females caged with experimental males for the resident-intruder test will be primed by subcutaneous injection of oestradiol to be behaviourally receptive while interacting with males.

Study 4: unravelling molecular mechanisms of social functioning
$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. The animals will be left undisturbed (except for cage cleaning) until ±24 week of age in which they will be sacrificed by rapid decapitation after CO_2 asphyxiation and brain tissue will be immediately collected and stored until the molecular analyses indicated in the previous section are performed.

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
$SERT^{+/+}$ and $SERT^{+/-}$ F1 rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1. ($SERT^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, $SERT^{+/+}$ rats will be used).

After ELS exposure, F1 rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype and ELS treatment. From 14 weeks of age (similar to study 1), we will test F1 affective-related behaviours by using the same tests used in study 1 (i.e., EPM, SPT and OFT) and following the same procedure. After the last behavioural test, F1 animals will be mated. To do so, depressed-like males and depressed-like females will be mated with wildtype, control females and males, respectively, to produce F2.

F1 pregnant females will be left undisturbed during pregnancy (except for cage cleaning). After delivery of F2, we will measure social communication of F2 pups on postnatal day 6 and maternal care of F1 females during the first postnatal week of F2.

Social communication of F2 pups will be measured by ultrasonic vocalizations produced by the pup in response to separation from the dam and littermates in a 5-minute test. The pup will be individually transported from the nest to a testing room in which no other animals will be present. The pup will be place in a Makrolon type 2 cage filled with Aspen wood chip that will be under the ultrasonic microphone.

Maternal care of F1 females will be tested by scoring care behaviours displayed by the dam towards the pups in the nest, three times a day -30 minutes each- during the first postnatal week of F2. Licking/grooming, arched-back nursing and contact with pups will be scored in the home cage to minimise any disruption to the dams (F1) or the offspring (F2).

F2 rats will be weaned at postnatal day 21 and socially housed with mates matched by experimental condition (see previous section about description of general design for further explanation of control and experimental conditions). From 14 weeks of age (similar to study 1), we will test whether the depressive-like phenotype is expressed in F2. Therefore, we will test F2 affective-related, cognitive, and social behaviours and molecular mechanisms involved by proceeding the same as we will proceed in study 1. After all behavioural tests have been taking place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

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

To estimate the sample size of each study, we used the Gpower 3.1 statistical software. The input parameters to calculate the sample size of each study for ANOVA (fixed effects, special, main effects, and interactions) statistical tests depended on three aspects: 1) the number of groups per study; 2) assumptions of power and alpha for statistical significance and, 3) the effect sizes reported in previous studies.

Aspect one: The number of groups required per study:

- Pilot study = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 1 = 16 groups (4 treatments, 2 SERT genotypes, 2 sexes)
- Study 2 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 3 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 4 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)

As in study 5 two generation of rats will be tested (i.e., F1 and F2), the estimation of animals for this study is as follow:

- F1 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes).
- F2 = 16 groups (4 parental conditions, 2 SERT genotypes, 2 sexes). See previous section about description of general design, study 5, for further explanation of control and experimental conditions of F2.

Aspect two: We based our calculations on the assumption to obtain a significant group effect with a power of 80% and alpha value of 0.05.

Aspect three:

For the pilot study, we used the effect size f=0,32 calculated from the finding of reduced sucrose preference induced by the ELS treatment reported by 5.1 lid2e, 5.1 lid2h [4], chapter 4. We chose this primary outcome because it is indicative of anhedonia, a key symptom in depression.

For behavioural studies 1, 2, 3, and 5, we used the effect sizes calculated from behavioural results reported by 5.1 lid2e, 5.1 lid2h [4], chapter 8. Specifically:

For studies 1 and 5, the effect size f=0,227 was calculated from the finding of reduced sucrose preference induced by the ELS treatment. We chose this primary outcome because it is indicative of anhedonia, a key symptom in depression.

For studies 2 and 3, the effect size f=0.253 was calculated from the finding of lower sociability induced by the ELS treatment. We chose this primary outcome because it is indicative of social withdrawal, also reported in depression.

Even though we do not know the behavioural outcomes of our proposed studies, especially of animals exposed to the cumulative ELS, we consider the effect sizes from findings reported by 5.1 lid2e, 5.1 lid2h as appropriate for our estimations as they also studied the interaction of ELS and the SERT genotype to test animals' behaviour and used several of the same tests we have proposed here.

For study 4, we used the effect size based on the findings reported by 5.1 lid2e, 5.1 lid2h [25]. Specifically, we used the effect size f=0,337 calculated from the finding of upregulated myelin-related gene expression in prefrontal cortex and downregulated myelin-related gene expression in basolateral amygdala of male rats exposed to ELS treatment. We chose this primary outcome because it indicates an effect of ELS exposure on molecular mechanisms of neural circuit connections involved in depressive-like behaviours.

Even though we do not know the molecular outcomes of animals exposed to ELS in our proposed study, we consider the effect size from findings reported by 5.1 lid2e, 5.1 lid2h appropriate for our estimations as they also studied the exposure to ELS as one factor influencing the gene expression and epigenetic regulation of some of the same brain regions we have proposed to analyse here.

Other considerations to calculate the number of animals:

1- Animals to be tested in all studies are born from mothers exposed to stress, therefore, we also estimated the number of females to be exposed to stress. This calculation is described in section B of this document, number of animals.
2- Animals to be used in studies 2 to 4 will be born from the same mothers. Therefore, we will assign 1 male and 1 female per litter to each study. On one hand, this will maximise the use of the offspring; on

the other, it will control litter effects for molecular analysis involved in studies 2 to 4. Even though we do not know the behavioural outcomes of studies 2 and 3, the brains for study 4 will be collected to optimise animal use (use of the same litters from studies 2 and 3).

Minimising the number of animals in studies 2 to 5 might be possible based on outcomes of study 1. If results from this study indicate that $SERT^{+/-}$ rats are not more vulnerable than $SERT^{+/+}$ animals, we will not use this knockout manipulation for studies 2 to 5; therefore, the number of animals needed for those studies might be reduced.

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
A2	Rattus norvegicus	Animals bred in our animal facility	Adult females to expose to stress and breed. Newborn, juvenile, and adult offspring to test behaviour and for molecular analysis	3382	Both	Yes	Wistar

Provide justifications for these choices

Species	Affective-related, cognitive and social behaviours in the rat resemble functional similarities of the same behaviours in humans. Similar to humans, rats are also highly sensitive to stress exposure very early in development. Changes in rat behaviour following *cumulative* ELS can resemble human behavioural changes after a long history of ELS. In addition, the brain circuit for the expression of affective-related, cognitive and social behaviours is highly conserved across mammals. Therefore, brain circuit functionality in the rat resembles brain circuit functionality in humans.

Origin	**Phase 1** **Pilot study:** F0 = SERT$^{+/-}$ males mated with SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0 **Phase 2** **Study 1:** F0 = SERT$^{+/-}$ males mated with SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0 **Studies 2 to 4:** F0 = SERT$^{+/-}$ males mated with SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups. This generation will be the parents of rats to be used in studies 2 to 4 F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0 **Study 5:** Origin of animals for control condition: F0 = SERT$^{+/-}$ males mated with control SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0, and mated with control SERT$^{+/+}$ rats to created F2 F2 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F1 Origin of animals for experimental conditions 1 and 2: F0 = SERT$^{+/+}$ males mated with stressed SERT$^{+/+}$ females F1 = stressed SERT$^{+/+}$ rats born from F0, and mated with control SERT$^{+/+}$ rats to create F2 F2 = SERT$^{+/+}$ rats born from stressed SERT$^{+/+}$ F1 rats Origin of animals for experimental conditions 3 and 4: F0 = SERT$^{+/-}$ males mated with stressed SERT$^{+/+}$ females F1 = stressed SERT$^{+/-}$ rats born from F0, and mated with control SERT$^{+/+}$ rats to create F2 F2 = SERT$^{+/+}$ rats and SERT$^{+/-}$ rats born from stressed SERT$^{+/-}$ F1 rats
Life stages	**Phase 1** **Pilot study** *Adult females:* for stress exposure and breeding at 3 months of age *Adult offspring:* for testing the depressive-like phenotype **Phase 2** **Study 1** *Adult females:* for stress exposure and breeding at 3 months of age *Adult offspring:* for testing the depressive-like phenotype and molecular analysis of brain tissue **Studies 2 to 4** *Adult females:* for stress exposure and breeding at 3 months of age *Juvenile offspring to study 2:* for social play testing and molecular analysis of brain tissue *Adult offspring to study 3:* for sexual behaviour and aggression testing and molecular analysis of brain tissue *Adult offspring to study 4:* for molecular analysis of brain tissue at the same age of rats used in study 3 to make outcomes of molecular analysis comparable **Study 5** *Adult females* (F0): for stress exposure and breeding at 3 months of age *Adult offspring* (F1): for testing the depressive-like phenotype and breeding *Newborns and adult offspring* (F2): for testing transgenerational behavioural effects of parental depressive-like phenotype and molecular analysis of brain tissue

Number

Phase 1

Pilot study

We will need n=10 animals per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 10 x 8 = 80. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed is 80 / 4 = 20. We estimated 10 pups delivered by each F0 female, so 20 x 10 = 200. If we use 2 male and 2 female pups per litter to get the total sample of 80 rats, 6 pups per litter will not be used (20 X 6 = 120). See calculations per treatment as follows:

F0 females exposed to ELS N=20		F1 rats used (2 males + 2 females per litter) N= 80		F1 rats exposed to ELS non-used for pilot study (6 pups per litter) N= 120	
Controls	Cumulative ELS	Controls	Cumulative ELS	Controls	Cumulative ELS
10	10	40	40	60	60

Exposure to stress in F0 females can interfere with normal body weight, affect their welfare, reduce the rate of fertilization, or alter pregnancy maintenance. Therefore, we estimated extra F0 females to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the cumulative ELS groups, we based our calculations according to the 1/3 of non-pregnancy rate reported by Gemmel et al [22]; hence we estimated 30% extra for cumulative ELS. In addition, extra F1 pups born from extra F0 females were also estimated to be used because stressed mothers might deliver fewer than 10 pups per litter, or animals born from stressed mothers might not survive due to impaired maternal care. See calculations of all extra rats per treatment as follows:

Extra F0 females for possible dropouts N=3		Extra F1 rats for possible dropouts (2 males + 2 females per litter) N=12		Extra F1 rats exposed to ELS non-used for pilot study (6 pups per litter) N=18	
Controls	Cumulative ELS	Controls	Cumulative ELS	Controls	Cumulative ELS
0	3	0	12	0	18

Phase 2

Study 1:

We will need n=14 animals per group; and we will have 16 groups (4 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 14 x 16 = 224. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed 224 / 4 = 56. We estimated 10 pups delivered by each F0 female, so 56 x 10 = 560. If we will use 2 male and 2 female pups per litter to get the total sample of 224 rats, 6 pups per litter will not be used (56 X 6 = 336). See calculations per treatment as follows:

F0 females exposed to ELS N=56				F1 rats used (2 males + 2 females per litter) N= 224				F1 rats exposed to ELS non-used for study 1 (6 pups per litter) N= 336			
Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3
14	14	14	14	56	56	56	56	84	84	84	84

Abbreviations: ELS-T1 = Postnatal stressors; ELS-T2 = Prenatal and postnatal stressors; ELS-T3 = Pregestational, prenatal and postnatal stressors.

Exposure to stress in F0 females can interfere with normal body weight, affect their welfare, reduce the rate of fertilization, or alter pregnancy maintenance, especially in females exposed to ELS-T3. Therefore, we estimated extra F0 females to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations according to the 1/3 of non-pregnancy rate reported by Gemmel et al [22]; hence we estimated 30% extra for ELS-T3. We estimated 15% extra for ELS-T2 and ELS-T1 each based on possible (although unlikely) reduced gestational length, litter size, or pre-weaning mortality [23]. In

addition, extra F1 pups born from extra F0 females were also estimated to be used because stressed mothers might deliver fewer than 10 pups per litter, or animals born from stressed mothers might not survive due to impaired maternal care (especially ELS-T3. See calculations of all extra rats per treatment as follows:

Extra F0 females for possible dropouts N=11				Extra F1 rats for possible dropouts (2 males + 2 females per litter) N= 44				Extra F1 rats exposed to ELS non-used for study 1 (6 pups per litter) N= 66			
Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3
0	3	3	5	0	12	12	20	0	18	18	30

Studies 2 to 4:
For study 2, we will need n=16 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 16 x 8 = 128.

For study 3, we will need n=16 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 16 x 8 = 128.

For study 4, we will need n=9 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 9 x 8 = 72.

Animals needed for studies 2 to 4 will come from the same litter and be born from dams (F0) exposed to stress. Therefore, the total number of F0 females needed is = 64. We estimated 10 pups delivered by each F0 female, so 64 x 10 = 640. If we will use 128 rats to the study 2, 128 rats to the study 3, and 72 rats to the study 4 (to get a total of 328 rats), the total of non-used animals will be =312. See calculations per treatment as follows:

	F0 females exposed to ELS N= 64		F1 rats used N=328		F1 rats exposed to ELS non-used for studies 2 to 4 N=312	
	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
Study 2	32	32	64	64	156	156
Study 3			64	64		
Study 4			36	36		

As in study 3 surgery for tubal ligation will be performed in females, we also estimated extra females to be used for possible dropouts related to this procedure. Therefore, if we will have 4 groups of females (2 treatments, 2 SERT genotypes), we estimated one extra female per group, therefore, n of dropouts=4

We also estimated extra F0 females and extra F1 pups to be used for possible dropouts for same reasons indicated above. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations assuming that the most robust ELS will be the ELS-T3; hence we estimated 30% extra. See calculations of all extra rats as follows:

	Extra F0 females for possible dropouts N= 10		Extra F1 rats for possible dropouts N=52		Extra F1 rats exposed to ELS non-used for studies 2 to 4 N=48	
	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
Study 2	0	10	0	20	0	48
Study 3			0	20		
Study 4			0	12		

The estimated number of companion females to test males' sexual behaviour is = 64
The estimated number of companion females to be housed with males for aggression test is = 64

Study 5:

Estimated number of F1 rats

We will need n=20 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 20 x 8 = 160. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed is 160 / 4 = 40. We estimated 10 pups delivered by each F0 female, so 40 x 10 = 400. If we will use 2 male and 2 female pups per litter to get the total sample of 160 rats, 6 pups per litter will not be used (40 X 6 = 240). See calculations as follows:

F0 females exposed to ELS N=40		F1 rats for behavioural testing and breeding N=70		F1 rats for behavioural testing only N=90		F1 rats exposed to ELS non-used for study 5 N=240	
Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
20	20	14	56	66	24	120	120

We also estimated extra F0 females and extra F1 pups to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations assuming that the most robust ELS will be the ELS-T3; hence we estimated 30% extra. See calculations of all extra rats as follows:

Extra F0 females for possible dropouts N=6		Extra F1 rats for possible dropouts (2 males + 2 females per litter) N=24		Extra F1 rats exposed to ELS non-used for study 5 (6 pups per litter) N= 36	
Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
0	6	0	24	0	36

Estimated number of F2 rats

We will need n= 14 rats per group; and we will have 16 groups (4 parental conditions, 2 SERT genotypes, 2 sexes); therefore, total sample size is 14 x 16 = 224. The parents of these animals will be F1 rats used for testing and breeding, indicated in the table above (N=70). We estimated 10 pups delivered by each F1 parent, so 70 x 10 = 700. If we will use 56 control animals and 168 experimental animals to get the total sample of 224 rats, n=476 will not be used. See all calculations below:

F2 rats used for behavioural testing and brain collection N=224		F2 rats born from F1 non-used for study 5 N= 476	
Controls	Experimental groups	Controls	Experimental groups
56	168	84	392

We also estimated extra F2 animals born from extra F1 rats because animals born from F1 depressed-like mothers might not survive due to impaired maternal care, or litters from SERT$^{+/-}$ parents might be smaller. See calculations as follows:

Extra F2 rats born from extra F1 animals N=96		Extra F2 born from extra F1 non-used for study 5 N= 144	
Controls	Experimental groups	Controls	Experimental groups
0	96	0	144

Sex	Depression in humans is expressed in both sexes, therefore we will conduct this research in male and female rats.
Genetic alterations	$SERT^{+/-}$ rats that express lifetime low expression of the serotonin transporter (SERT) will resemble low SERT expression in human carriers of the short allele in the SERT gene.
Strain	Wistar

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.

- In study 3, one or two tail incisions (in case taking off the crust from the first incision does not work) will be made to collect the blood as described in section A of this form. Pain relieving will not be used for this procedure because the severity is mild and, if properly executed, it is not expected to cause any detectable adverse effect.
- Studies 1 and 4, and pilot study involve brain tissue collection after decapitation to perform molecular analysis. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible. Anaesthesia is not recommended before decapitation because it can alter the analysis of gene expression.

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

- Studies 2 and 3 involve brain tissue collection after perfusion to perform analysis of c-Fos expression. Preparation for perfusion will involve an intraperitoneal overdose of pentobarbital for deep anaesthesia and non-recovery. The dose will be adjusted for each animal's weight.
- For double tubal ligation to females in study 3, each female will be under anaesthesia. To minimise discomfort directly linked to the surgery, analgesics will be subcutaneously injected. The female will receive analgesics 24 hrs and 48 hrs hours after surgery. During the 14-day recovery period, the weight and the well-being of the animals will be daily checked.

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

- Chronic unpredictable stress applied to females before conception may reduce their body weight and/or interfere with fertilization.
- Restraint stress in pregnant females may affect their body weight, the maintenance of pregnancy, the number of offspring alive after delivery and/or litter size at birth.
- Unpredictable maternal separation and disrupted maternal care may potentially increase pups' mortality and/or may reduce the body weight of both mothers and the litter.
- Isolation of females 1 week prior to female-resident intruder test may induce stress (study 3)

Explain why these effects may emerge.

- Prolonged exposure to stress dysregulates the release of glucocorticoids. This interferes with the normal activity of glucose, thus affecting the metabolism of animals. In addition, high levels of glucocorticoids induced by stress can interfere with normal functioning of sexual hormones associated with embryo implantation.

- Exposure to high levels of stress during pregnancy increases the levels of glucocorticoids into systemic circulation that can interfere with the functioning of the placenta or foetal development. This situation can alter the maintenance of gestation, normal foetal growth, or number of pups alive after delivery.
- Separation from mothers or disrupted maternal care may reduce the amount or quality of mothers-litter nurturing, therefore, these procedures may reduce the amount or quality of nesting, lactation, or mothers' licking behaviour towards their litters, all of which are necessary for pups' development.
- Social isolation is stressful for social animals.

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

- We will do 3 attempts of rebreeding (maximum) as soon as the pregestational stress procedure is over. Pregestational stress procedure can finish 0-2 weeks before the gestational period begins without interfering with the experimental outcome [26].
- We will provide sufficient food and water ad libitum to maintain body weight as normal as possible.
- We will provide enough bed material, gnawing sticks and group housing for environmental enrichment that helps to cope with stress.
- We will provide food and water ad libitum, enough bed material and gnawing sticks for environmental enrichment for isolated females in study 3.
- We will monitor that animals are not exposed to sources of stress other than stressors used for the experiments. This means we will prevent a noisy environment, inadequate room temperature, light, or ventilation, inadequate handling, etc.

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.

If animals show clear signs of the sickness behaviour and/or display grimace signs of pain (arched back, rough coat, general malaisse) or loose more than 15% of their body weight in 48 hours, they will be excluded from the experiment and terminated. If they show tumors, they will be terminated. We will not interfere with the pups until they are weaned (that is, outside the maternal separation, we will leave the nest with the mom).

Indicate the likely incidence.

Very low. Although stress procedures are chronic, our previous experience as well as research conducted elsewhere have shown that the level of discomfort induced by selected stress procedures do not significantly interfere with feeding, water consumption, motor activity, or cleaning behaviours. Based on van den Hove et al [23] procedure, prenatal stress had no effect on gestational length, litter size, or pre-weaning mortality, so restraint stress effects on pregnant rats are very unlikely. As far as we know, nobody has performed studies combining pregestational, prenatal and postnatal ELS, therefore, we are not sure about the incidence of negative effects. With drug treatments (maternal fluoxetine use) we used before we saw viability index of 72-73%. We expect these stressors to have a higher viability index as we saw that only one ELS had no significant effects [Chapter 5 in ref 4]. If 28% would die, we still have enough animals left in the litter. We will monitor the welfare of the females and offspring to properly record it and take actions to implement humane endpoints if applicable.

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).

For the pilot study, we expect the pregestational, prenatal and postnatal stressors to be in the range of moderate. Especially because in our hands, the pre- and postnatal stressors have never led to a higher discomfort than 'moderate'. The combination of all three stressors is new, and we will monitor the animals closely and will contact immediately the Institutional Animal Care and Use Committee when the discomfort will be higher than anticipated (or when in doubt). Experiments will end (in consultation with the IACUC) when they exceed "moderate" discomfort levels. The animal behavior tests that the animals will undergo, are similar to what we have performed before in our lab and are in the range of mild. Cumulative discomfort for this group will therefore be estimated as 'moderate'.

For all other groups, the behavioral tests we will use are tests we have ample experience with. In our hands, they never exceeded 'moderate' discomfort levels. The early life stressor with the most robust effects will be used, which may mean the pregestational, prenatal and postnatal stressors might be picked as the preferred model. We expect the discomfort of the mother to have an impact on the offspring, however we still expect this to be in the 'moderate' range when combined with the behavioral experiments (which are mild to moderate).

Of a total of estimated animals to be used, it is expected that 22,5% of animals are exposed to cumulative mild discomfort and 77,5% are exposed to cumulative moderate discomfort.

	All studies		Pilot study & Study 1		Study 2		Study 3		Study 3		Study 4		Study 5		Study 5		Companion females housed with males + stimulus females to test males' sexual behavior in study 3	All studies		Study 5	
	F0		F1		F1		F1 males		F1 females		F1		F1		F2			Non-used F1 rats		Non-used F2 rats	
	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	EXP		CTR	ELS	CTR	EXP
Arrival	2	2																			
Handling	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2				
Stress exposure		3		3		3		3		3		3		3					3		
Cognitive tests			2	2											2	2					
Affective tests			2	2									2	2	2	2					
Social tests			2	2	2	2	3	3	3	3					2	2					
Home accommodation to induce territorial behaviour							2	2	3	3											
Repeated blood sampling							3	3	3	3											
Tubal ligation surgery									3	3							3				
Primed s.c. oestradiol treatment (max 1x every two weeks)																	2				
Euthanasia	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
Cumulative discomfort (SV)	SV2	SV3	SV3	SV3	SV2	SV3	SV3	SV3	SV3	SV3	SV2	SV3	SV2	SV3	SV3	SV3	SV3	SV2	SV3	SV2	SV3
% of animals to have expected cumulative discomfort	2,2	4,0	2,8	7,8	1,9	2,5	1,0	1,2	1,0	1,3	1,1	1,4	2,4	3,1	1,7	7,8	3,8	12,4	22,3	2,5	15,8

***** *EU directive scale: SV1=terminal, SV2=mild, SV3=moderate, SV4=severe*

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	The measurement of the depressive-like phenotype in the rat model implies the assessment of complex patterns of behaviours to resemble the human condition. *In vivo* studies conducted with rats allow to investigate a wide range of behaviours that are functionally similar

	to those observed in humans, as well as the underlying mechanisms in the brain. Additionally, the functioning of brain regions involved in regulation of affective-related, cognitive, and social behaviours in humans is highly conserved in rats and cannot be found in lower organisms; therefore, the use of these types of live animal experiments is still irreplaceable. Moreover, *in vitro* studies on isolated tissue would not be able to give a sufficient overview of the effect of *cumulative* ELS in the brain and behaviour in the offspring. In short, it is not feasible to replace the use of live animals in this research project.
Reduction	To assess the effects of cumulative ELS and SERT gene interaction, intact and freely behaving rats will have to be used. We have developed in-depth expertise in the proposed animal experimental paradigms; in addition, we have selected validated, optimised, and refined protocols that will reduce the number of animals needed to obtain significant results. The number of animals needed for these studies will be carefully considered based on prior studies and expected variance in the dependent variables. Furthermore, we consider carefully which models are needed for our studies. First, the go/no go moments are described in the section 3.4.1 of the proposal form. If findings from the pilot study do not show behavioural primary outcomes of a depressive-like phenotype in rats exposed to cumulative ELS, conducting phase 2 will no longer be relevant. Furthermore, if $SERT^{+/-}$ rats do not express higher vulnerability to depressive-like behaviours than $SERT^{+/+}$ rats, they will no longer be used in studies of phase 2. Hence, only $SERT^{+/+}$ rats will be included and the number of groups and animals in studies 1 to 5 would be reduced. Second, estimated number of animals per group per study is based on statistical methods aimed at minimising the number of animals. Third, the offspring of studies 2 to 4 will come from the same parents, thus maximising the use of the offspring.
Refinement	All procedures are used regularly in our laboratory and have been previously refined to minimise the potential discomfort. The responsible researchers have ample expertise with these procedures and with training other researchers. The animal housing facilities are well-equipped to house rodents. The responsible researchers and the staff from the animal facilities are properly trained to handle the animals; assess the health and welfare of the animals; administer anaesthesia and minimise pain and suffering. All surgical procedures will be performed under anaesthesia, with proper post-operative care. Animals will be humanely killed at defined end points according to national ethical rules. Stress procedures will produce discomfort in animals. However, ad libitum food and water (unless stated otherwise) and environmental enrichment through social housing, gnawing sticks, and sufficient nesting material in the home cage in all studies will be ensured to increase strategies to cope with stress.

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.

In study 5, non-stressed F1 rats can be re-used for educational purposes as discomfort in them is classified as mild. However, if they are declared unfit for further use by the designated veterinarian (Art 14), killing will be done according to EU guidelines. Animals will only be re-used if they, at the end of the study, are suitable for other experiments covered by an existing CCD license or studies that are below threshold; if not, we will sacrifice them.

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

For studies 1 and 4 and pilot study, killing by decapitation is required for brain collection. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible.

For studies 2 and 3, killing by perfusion is required for brain collection. Intraperitoneal overdose of pentobarbital for deep anaesthesia and non-recovery will be used.

For study 5, F1 animals will be sacrificed after completing the behavioural tests and/or breeding as they have fulfilled the scientific purpose and their cumulative discomfort is classified as moderate. F2 animals of study 5 will be killed by decapitation for brain collection. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible.

Companion females will be sacrificed after completing study 3 as they have fulfilled the scientific purpose and cumulative discomfort is classified as moderate.

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 will be used in F0 rats and non-used animals of all studies. It will be used also in companion females and F1 rats of study 5. The animal will be transported to a gas chamber in which CO_2 flow will be allowed until pressumed death is confirmed.
☒ Yes > Describe the method of killing that will be used and provide justifications for this choice.
Decapitation will be used in animals of the pilot study and studies 1, 4 and 5 to collect brain tissue and analyse gene expression level and epigenetic markers. Decapitation is selected because fresh frozen tissue samples are preferred to analyse gene expression level by using the quantitative polymerase chain reaction (RT-qPCR) technique. Prior to decapitation, the rat will be taken to a different room and will be exposed to CO_2 asphyxiation produced from dry ice until sedation. Immediately after, the animal will be decapitated by using a guillotine. Perfusion will be used in animals of studies 2 and 3 to collect brain tissue and analyse the c-Fos expression level. Perfusion is preferred to maximise good quality of brain slices to perform immunohistochemistry staining for c-Fos quantification. Prior to perfusion, the rat will be anaesthetised with an overdose of pentobarbital. Immediately after, a transcardiac perfusion with saline followed by 4% paraformaldehyde will be performed to clear blood and preserve the brain tissue.
If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

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8

Van: info@zbo-ccd.nl
Verzonden: vrijdag 10 november 2023 16:33
Aan: 5.1 lid2e ;5.1 lid2h
CC: 5.1 lid2e 5.1 lid2h
Onderwerp: Aanhouden AVD5.1 lid2h202317174

Categorieën: Dossier: 5.1 lid2e

Geachte 5.1 lid2e ,

Op 04-07-2023 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Unravelling the underlying mechanisms of early life stress induce disorders in animals with expression of the serotonin transporter" met aanvraagnummer AVD5.1 lid2h202317174. 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
- De NTS bevat over de gehele tekst en titel vaktermen (zoals, maar niet beperkt tot, neurotransmitter, postnatale, hersencircuit), waardoor de NTS voor het algemene publiek lastig navolgbaar kan zijn. Kunt u uw NTS nalopen op dergelijke termen en deze termen aanpassen of uitleggen?

- In de bijlage dierproeven geeft u aan een pilotexperiment te zullen uitvoeren om de proefopzet van het hoofdonderzoek te optimaliseren. Kunt u het uitvoeren van het pilotexperiment opnemen in uw NTS?

- In de bijlage dierproeven geeft u aan dieren individueel te huisvesten bij het afnemen van een ‘resident-intruder’ test. Het individueel huisvesten van dieren kan tot ongerief leiden. Kunt u individuele huisvesting opnemen in uw NTS?

- In de bijlage dierproeven geeft u aan ratten te onderwerpen aan voederrestrictie. Het onderwerpen van dieren aan voederrestrictie kan tot ongerief leiden. Kunt u voederrestrictie opnemen in uw NTS?

- In de NTS spreekt u over procedures waarin dieren cumulatieve stress krijgen. Kunt u inzichtelijk maken in uw NTS waar de dieren stress van krijgen?

Onduidelijkheden
- Kunt u de beantwoording van 3.2.3 opnemen in uw projectvoorstel?

- Onder C. in uw bijlage dierproeven geeft u aan niet af te wijken van de richtlijnen beschreven in Annex III van de EU richtlijn. Kunt u hier het individueel huisvesten van ratten evenals de voerrestrictie opnemen in de toelichting?

- Onder F. in uw bijlage is het individueel huisvesten van vrouwelijke ratten tijdens de pilot studie niet opgenomen in de ongeriefsclassificatie. Kunt u dit opnemen onder F. en het cumulatieve ongerief (indien noodzakelijk) 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.

Mochten uw antwoorden voor vrijdag 17 november a.s. door ons ontvangen zijn, kunnen deze worden meegenomen tijdens de inhoudelijke bespreking van uw dossier in de vergadering van de CCD.

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

.....
Postbus 93118 | 2509 AC | Den Haag
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Centrale Commissie Dierproeven

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see the Guidelines to the project licence application form for animal procedures on our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0800-7890789).

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.	Transgenerational susceptibility to depression due to early life stress

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.

Depression is a mental health condition characterised by sadness, loss of interest or pleasure, feelings of guilt and low self-worth that substantially impairs an individual's ability to cope with daily life. Cognitive impairments, altered affective behaviour, and social disfunctions are often present in depressed

individuals [1-3]. Depression is the most prevalent neuropsychiatric disorder and the first cause of disability in the world [4]. The social burden related to depression is steadily increasing, and the numbers for depressive disorders may increase even more in the next few years due to the unprecedented negative effects of the COVID-19 pandemic on mental health.

Women suffer from depression twice as often compared to men [5] and unfortunately pregnant women are not spared. Approximately one out of five pregnant women suffer from depressive symptoms [6], which can have long-term health consequences in the offspring [7]. Early life programming through an adverse intrauterine environment, or postnatally, increases susceptibility to a myriad of diseases, including psychiatric disorders [8]. Thus, an adverse early life environment increases the risk for depression. However, this risk is increased when a subject has vulnerable genes as was shown by the seminal paper of Caspi et al. [9]. This, and later studies identified the critical interaction of genetic and environmental factors that contribute to depression [10-13]. Caspi and colleagues found that the risk for depression increased when the number of stressful life events that people encountered increased. Additionally, they studied the extent to which the genotype that regulates the serotonin transporter (SERT) expression moderates the influence of stress on depression. In the serotonin-transporter-linked promoter region (5-HTTLPR), different lengths of the repetitive sequence containing GC-rich, 20-23-bp-long repeat elements in the upstream regulatory region of the gene have been identified. Deletion or insertion in the 5-HTTLPR is referred to as the 14-repeat short (S, low expressing) and the 16-repeat long (L, high expressing) alleles. Caspi and colleagues found that individuals who were carriers of one or two copies of the S-allele were more vulnerable to developing depression following stress exposure than carriers of the L-allele exposed to stress [9].

Many studies reproduced the Caspi's research, although a large genome-wide study found no association between the SERT genotype and increased risk for lifetime prevalence of depression in people exposed to stress [14]. Also, rodent studies fail to show solid evidence for increased vulnerability to developing depressive-like behaviour after early-life stress (ELS) in rodents with reduced SERT (heterozygous; $SERT^{+/-}$) expression [15]. Therefore, the potential association of the SERT gene and psychiatric condition remains inconclusive. Nevertheless, previous studies show that the influence of 5-HTTLPR can be heterogeneous and highlight possible involvement of other factors and regulatory mechanisms promoting the risk of psychiatric disorders [16,17]. Recently a study in an elderly Lithuanian population showed that a 5-HTTLPR × lifetime stressful events interaction effect on depression was observed [18]. The highest odds of depression were found in participants with both high stress and s/s genotype for life time stressful events.

What has been consistently shown is that ELS exposure has a large impact on mental health later in life [19,20], and that ELS is not restricted to stress exposure during childhood (postnatal stress), but can also include exposure to stress during the foetal period, mediated by a mother that is stressed during pregnancy or even before conception [21,22]. Considering pregestational stress, it was shown that physiological alterations (flattened child diurnal cortisol slopes) take place when posttraumatic stress disorder was diagnosed before pregnancy [23]. Thus, ELS to the offspring can take place by 1) maternal stress/depression before pregnancy (*pregestational*; which is transferred to the offspring during pregnancy); 2) maternal stress/depression during pregnancy (*prenatal*; transferred to the offspring during pregnancy); and 3) either maternal stress/depression, or direct stressors to the offspring after birth (*postnatal*; during the first years of life).

With this additional evidence of maternal stress mediating offspring long-term outcomes, the studies about the ELS x SERT genotype interaction can be revisited. One of the reasons that the ELS x SERT studies are inconclusive may be because sustained maternal stress, starting before child's birth and even before conception, has not been taken into account. Moreover, clinical research supports the notion that neuropsychiatric disorders, including depression, have a developmental origin mediated by the conditions of the mother before the conception of the child [21]. From this view, the exposure to *cumulative* ELS can interact with the SERT genotype -of the offspring- to increase the risk of depression. To our knowledge, no one has studied whether the onset of depression in the adult offspring is mediated by the interaction of *cumulative* ELS exposure and the SERT genotype. The study published by Tiemeier et al.

[24] strongly suggest the importance to do so. They found that maternal anxiety -that involves stress in the mother- during pregnancy and postnatally increased the risk of child emotional problems and leads to less accurate emotional matching in 3-year-old S-allele carriers. Of interest is the systematic review and meta-analysis of Delli Colli and colleagues who show that a 5-HTTLPR gene x environment x time interaction exist [25]. When depression is measured within a year after chronic stress a significant interaction with the 5-HTTLPR and stress was found, an effect that was not found after an acute stressor. The effect was also only found when the subjects were tested within 1 year after the stressor, and not when the stressor happened before the last year. However, what the authors fail to mention is that in the acute stressor group only stressors during adulthood took place, while in the chronic stress group 9 out of the 13 included studies involve early life stress. The question that rises, is whether the significant effect was found due to timing, or due to the fact of early life stress (or both). This is still not clear, and needs clarification.

There are ethical and methodological limitations to control the timeframe and type of *cumulative* ELS exposure that leads to elucidate its role in the adult onset of depression in S-allele carriers. Fortunately, valid animal models can help to study this association. Rodents do not express the 5-HTTLPR; however, heterozygous SERT knockout rodents ($SERT^{+/-}$) show neurochemical similarities to human S-allele carriers [26,27]. From a previous research project of our group, we provided a comprehensive overview of the behavioural effects of ELS in $SERT^{+/-}$ rodents and the neurobiological mechanisms involved [15]. We found that studies of postnatal ELS in $SERT^{+/-}$ rodents failed to show solid evidence for increased vulnerability to a depressive-like phenotype when $SERT^{+/-}$ adult animals were tested. We also performed a study in $SERT^{+/-}$ female rats to test whether their exposure to postnatal ELS increased their vulnerability to depressive-like behaviours when they were adults. The average of $SERT^{+/-}$ females did not exhibit consistent behavioural changes of depressive-like responses after ELS, although some females exposed to postnatal ELS showed increased affective-related behaviours [28]. In light of our previous experiments where dams were subjected to early life stress (ELSD) and their offspring behaviour was tested, we saw that $SERT^{+/-}$ male offspring showed reduced anxiety and depressive-like behaviour [29], an effect not found in females, nor in $SERT^{+/+}$ rats. This implicates a higher sensitivity in $SERT^{+/-}$ rats to pre-gestational stress, but outcomes are beneficial. Regarding social behaviours, we found no ELSD x genotype effects, although $SERT^{+/-}$ offspring in general engaged less in social interaction [30]. An ELSD x genotype interaction was found in aggressive behaviour, with $SERT^{+/-}$ rats showing increased offensive behaviours compared to their controls [31]. One explanation for not finding large effects in affective behaviours could be explained by the fact that the ELSD we applied did not induce robust depressive-like behaviour in the mother. In our first study, we found a lower sucrose preference in females exposed to the early life stressor [32], however, we couldn't replicate this effect in a later study [28]. When testing the offspring of ELSD, we did not test the mothers for depressive-like behaviours. Therefore, it is hard to tell whether all mothers showed a depressive-like effect. Of interest though is the recent study of Woo et al., 2023 [33] who showed similar effects after pregestational stress in $SERT^{+/-}$ mice. When chronic variable stress was applied during pregnancy, male offspring showed reduced anxiety levels, but also reduced social preference. This study, together with our studies, points to the direction that males might be more vulnerable to ELS in respect to social aspects, but not the affective aspects of behaviour. It is therefore important to test both of these behaviours in both sexes.

Considering the findings of our previous research project, postnatal ELS is not optimal or severe enough to induce a depressive-like phenotype in $SERT^{+/-}$ rodent models. In the present research project, we will investigate whether $SERT^{+/-}$ male and female rats are more vulnerable to develop a depressive-like phenotype in adulthood following the exposure to *cumulative* ELS. Moreover, this project will help us understand the underlying mechanisms of SERT x *cumulative* ELS interactions in the onset of depression later in life.

Based on our findings and the literature, we propose to apply pre-gestational, prenatal and postnatal stressors and investigate how these stressors cumulatively affect wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ offspring (both male and female). Not only are we interested in the mechanisms underlying this multiple stressor system, but also whether the SERT gene expression level increases the vulnerability to

depression following *cumulative* ELS. This will prevent using a high number of animals in the future as we could select more animals expressing a depressive-like phenotype after a single manipulation of multiple ELS stressors and use this for instance as a model for maternal stress.

We will study cognitive, affective-related and social behaviours, as well as gene expression and epigenetic markers (study 1). We will also characterise the social functioning and neurobiological mechanisms involved in studies 2 to 4. Lastly, we will test whether the offspring of the depression-like parents also express a depressive-like phenotype, as well as the extent to which epigenetic markers are shared in both generations (study 5).

In summary, it is critical to get fundamental insight in how SERT and ELS interact to induce stress-related disorders, not only to understand the mechanisms underlying these vulnerabilities, but also to pave the way for further research into new treatment targets of (maternal) depression. Because of the mechanistic approach in this research this is considered basic research. We hypothesise that a cumulative history of ELS exposure will increase the risk of disrupted cognitive, affective-related, social behaviours in the offspring. In addition, we hypothesise that animals with a vulnerable genetic background ($SERT^{+/-}$ rats) will be more vulnerable to develop depressive-like behaviours (or in a more severe fashion) than those with a non-vulnerable genetic background. Finally, we hypothesise that the effects of stressful early-life experiences of parents will be transferred to their offspring through inheritance of epigenetic markers.

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 research is to determine whether the interaction of *cumulative* ELS and the SERT genotype increases the vulnerability to developing a depressive-like phenotype and to what extent the parental depressive-like phenotype is transferred to the next generation. Neurobiological mechanisms -including epigenetic markers-, depressive-like behaviours -including affective-related, cognitive and social behaviour- and transgenerational effects will be elucidated in the rat model.

To reach this ultimate goal, immediate goals are proposed to be attained by conducting six studies:

Phase 1: Pilot study
Goal 1: To determine whether the combined exposure to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS, ie *cumulative* ELS, induces a depressive-like behavioural phenotype in the rat.
Goal 2: To determine whether the $SERT^{+/-}$ genotype increases the risk for depressive-like behaviours after the exposure to *cumulative* ELS.

Phase 2: Studies 1 to 5
Goal 3: To assess the neurobiological mechanisms involved in the depressive-like phenotype induced by *cumulative* ELS.
Goal 4: To identify whether social functioning and its neurobiological mechanisms are altered in young and adult rats exposed to *cumulative* ELS.
Goal 5: To determine the extent to which the depression-like phenotype induced by *cumulative* ELS in one generation is transferred to the next and evaluate the neurobiological mechanisms involved.

3.2.2 Provide a justification for the project's feasibility.

Our research group has ample experience conducting behavioural experimentation to assess cognition, affective-related and social behaviours in rodents, especially also in serotonin transporter knockout animals (SERT). The SERT animals have been bred at our facility for many years, making the project

feasible. In addition, our research group has experience in performing molecular analyses (microarray, qPCR, genotyping, DNA methylation, DNA hydroxymethylation, histone methylation).

We do not expect technical difficulties in conducting the experiments as we already have experience in studying ELS effects on brain development and behaviour of transgenic models.

All equipment and housing needed for experiments are available at the institute to which our research group belongs.

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?

This project addresses the influence of an environmental factor interacting with a genetic factor in the development of stress-related disorders. It also provides mechanistic insights in the effects of *cumulative* ELS on the development of stress-related disorders, especially in relation to the serotonin transporter genotype. Good animal models are indispensable in this research, as we can invasively study the underlying mechanisms in the brain of animals exposed to ELS mediated by the maternal stress (pregestational and prenatal), something that is not possible in humans.

If the outcome shows that more rats express a depressive-like phenotype as a result of *cumulative* ELS, we will be able to elucidate the underlying mechanisms contributing to stress-induced disorders. In addition, if $SERT^{+/-}$ rats exhibit higher vulnerability to developing a depressive-like phenotype following the *cumulative* ELS exposure, we will be able to demonstrate that the serotonin transporter genotype moderates the influence of stress on depression. This would greatly benefit the clinical management of stress-induced disorders as new targets for drug treatment may be revealed.

Additionally, if altered social behaviours are observed in adult and especially also in young animals exposed to *cumulative* ELS, we will be able to show a different neurodevelopmental trajectory of social functioning induced by *cumulative* ELS presumably related to the late onset of depression.

Lastly, by studying if the parental depressive-like phenotype induced by *cumulative* ELS is transferred to the next generation, we will provide evidence of epigenetic signatures of the depressive-like phenotype shared between parents and the offspring.

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

Our research group along with other researchers conducting basic research on stress-related disorders, including depression, can use this animal model to further investigate neurobiological underpinnings related to how *cumulative* ELS influences the development of the brain to induce depressive-like behaviours, both separately and in combination with the SERT genotype. This model would also allow further investigation of the mechanisms underlying transgenerational effects of depression and the onset of social dysfunctions related to depression.

In the long-term, patients with depression might benefit from our studies when it will reveal new treatment targets.

Also in the long term, pharmaceutical companies focused on developing pharmacological agents may be interested in this animal model to test the efficacy of different drugs in treating depressive-related symptoms.

As experimental subjects, the condition of rats as stakeholders is done under the principles of the 3Rs and setting go/no-go moments. The refinement of depression models (cumulative ELS and/or SERT genotype) can lead to a decrease in the number of animals used for this purpose in the future.

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.

Phase 1: Pilot study

We will expose wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ rats to *cumulative* ELS to test whether they develop depressive-like behaviours when they are adults.

For this research project *cumulative* ELS refers to the combination of (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS exposure (see appendix 1 to this form for further description of each ELS type). $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to this *cumulative* ELS will be compared with $SERT^{+/+}$ and $SERT^{+/-}$ control animals.

We will test affective-related behaviour, cognitive performance and social functioning to obtain a comprehensive behavioral phenotype of the depressive-like rat model.

Milestone #1: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses of rats exposed to *cumulative* ELS compared to rats not exposed to ELS.
Milestone #2: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses induced by *cumulative* ELS are significant more impaired in $SERT^{+/-}$ rats compared to $SERT^{+/+}$ rats.

Selection points and decision criteria

The design and execution of phase 2 will be based on how the questions in the black squares below are solved according to findings in the pilot study:

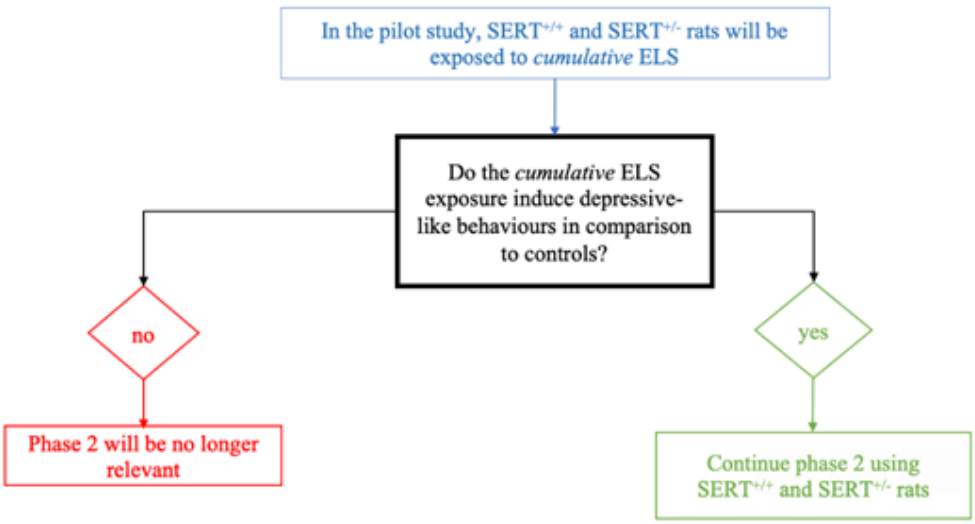

Fig1. Decision criteria to conduct phase 2 based on findings of phase 1. ELS: Early life stress; SERT: Serotonin Transporter.

If depressive-like behaviours are not expressed in animals exposed to *cumulative* ELS in comparison to controls in the pilot study, we will not continue to phase 2. If depressive-like behaviours are induced by cumulative stress, we will continue with phase 2 with both $SERT^{+/-}$ and $SERT^{+/+}$ rats.

Phase 2

Study 1: the depressive-like phenotype by *cumulative* ELS

We will expose $SERT^{+/+}$ and $SERT^{+/-}$ rats to *cumulative* ELS, to investigate whether they develop depressive-like behaviours when they are adults after being exposed to different combinations of ELS. Four conditions, one control and three experimental, will be compared in this study:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition 1: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to postnatal ELS (ELS treatment 1).
Experimental condition 2: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) prenatal ELS and postnatal ELS (ELS treatment 2).
Experimental condition 3 (same as pilot study): $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS (ELS treatment 3).

We will test affective-related behaviour, cognitive performance and social functioning in adult rats. We will also analyse mRNA expression level of selected genes, and epigenetic markers of those genes, in brain regions that are known to be involved in depression. Selected genes will at least include those involved in the serotonergic system, brain stress system (HPA axis), myelination and neural growth and survival as many of them can be co-expressed in major neural circuit connections involved in depressive-like behaviours in rodents [34]. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures and molecular analyses.

Milestone #1: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses of rats exposed to *cumulative* ELS compared to rats not exposed to ELS
Milestone #2: increased anxious behaviour or decreased depressive-, cognitive- or social behavioural responses induced by ELS are significant more impaired in $SERT^{+/-}$ rats compared to $SERT^{+/+}$ rats.
Milestone #3: altered gene expression in brain tissue of rats exposed to experimental stress conditions compared to controls.
Milestone #4: altered DNA methylation (correlated to gene expression) at the promotor regions of selected genes in brain tissue of rats exposed to experimental stress conditions compared to controls.

Selection points and decision criteria

The design and execution of studies 2 to 5 will be based on how the questions in the black squares below are solved according to findings in study 1:

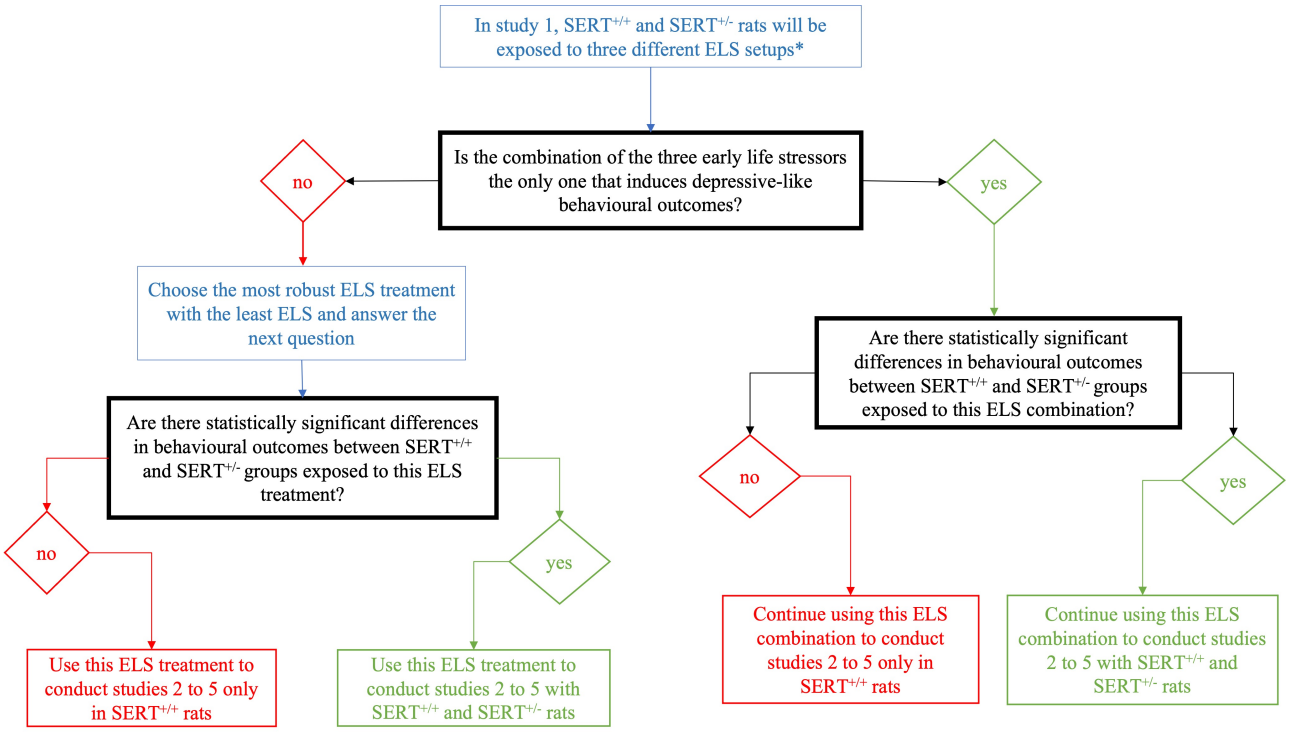

Fig2. Decision criteria to conduct studies 2 to 5 based on findings of study 1. ELS: Early life stress; SERT: Serotonin Transporter. *The three ELS treatments refer to the experimental conditions 1, 2 and 3 indicated in the text above (page 7).

Based on study 1, we will determine if the *pregestational, prenatal and postnatal* ELS is the most robust treatment to induce a depressive-like behavioral phenotype over the other two ELS treatments, and we will use it to the following studies. If not, we will select the ELS treatment that most robustly induces the depressive-like phenotype, with the least stressors, to use in the following studies and will discard the others. In addition, if $SERT^{+/-}$ rats do not express more vulnerability to depressive-like behaviours than $SERT^{+/+}$ rats, then $SERT^{+/-}$ rats will no longer be used in studies 2 to 5, and only $SERT^{+/+}$ animals will be included.

Study 2: focus on offspring social functioning in the juvenile period
We will expose $SERT^{+/+}$ and (depending on study1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. Next, we will compare social play behaviour between them when they are juveniles (both males and females). After the social play test, we will collect the brain tissue to analyse neuronal activity – through c-Fos expression by immunohistochemistry- in brain regions that regulate social play behaviour (e.g., prefrontal cortex, dorsal and ventral striatum, amygdala, habenula; [35]). See appendix 1 to this form for detailed description of the general design of the animal procedures.

Milestone #1: reduced social play behaviour and altered c-Fos expression in prefrontal cortex, dorsal and ventral striatum, amygdala, or habenula, all brain regions that regulate this behaviour between animals exposed to the ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Study 3: focus on offspring social functioning in adulthood
We will expose $SERT^{+/+}$ and (depending on study1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. When animals are adults, we will assess sexual behaviour and aggressive behaviour in both males and females. After the last aggression test, we will collect the brain tissue to analyse neuronal activity -through c-Fos expression by immunohistochemistry- in regions of the social brain network that regulate this behaviour (e.g., medial preoptical area, periaqueductal grey

matter, ventromedial hypothalamus, medial amygdala, [36]). See appendix 1 to this form for detailed description of the general design of the animal procedures.

Encountering intruders in their own territory induce a stress response in male and female rodents [37]. One way to measure this response is by assessing corticosterone (the main stress hormone in rodents) after the rat encounters an intruder in his/her territory [37,38]. Hence, when we test aggressive behaviour, we will take the aggression test as an opportunity to evaluate if corticosterone levels are differently affected by ELS treatment, SERT genotype, or its combination when the rat encounters an intruder in his/her own home cage.

Milestone #1: decreased sexual and aggressive behaviours between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).
Milestone #2: altered c-Fos expression after the aggression test, in brain areas that regulate aggressive behaviour between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).
Milestone #3: altered corticosterone levels following the aggression test between rats exposed to the most robust ELS treatment and controls, and between $SERT^{+/+}$ and $SERT^{+/-}$ rats (if this group is included).

Note: detailed procedures to test social play, sexual and aggressive behaviours and c-Fos expression in brain regions related to these behaviours are described in the appendix 1 of this proposal, section A, studies 2 and 3, respectively. Supported literature is provided correspondingly.

Study 4: unravelling molecular mechanisms of social functioning
We will evaluate the mRNA expression for selected genes, and epigenetic markers of those genes, in brain regions of the social brain network [35]. We will expose $SERT^{+/+}$ and (depending on study 1) $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. Next, animals will be left undisturbed until they are ±24 weeks old, when we will collect their brain tissue. This time of brain tissue collection is selected to be similar to the time in which we will collect the brain tissue in study 3. Selected genes will at least include those that code for vasopressin, serotonin, corticoid and oxytocin receptors as many of them can be co-expressed in several regions of the social brain network. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures.

Milestone #1: Altered gene expression in brain areas important for social behaviour in rats exposed to ELS compared to controls.
Milestone #2: altered DNA methylation at the promotor regions of selected genes in brain regions selected for social behaviour in rats exposed to ELS compared to controls.
Milestone #3: significant different gene expression/ DNA methylation in $SERT^{+/-}$ rats compared to $SERT^{+/+}$ rats after ELS.

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
We will determine if the depressive-like phenotype in one generation is transferable to their offspring. To accomplish this, first we will induce a depressive-like phenotype by exposing $SERT^{+/+}$ and $SERT^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. ($SERT^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, only $SERT^{+/+}$ rats will be used).

When F1 are adults, we will test their affective-related behaviour to confirm that the depressive-like phenotype is expressed. Then, depressed-like F1 males and depressed-like F1 females will be mated with wildtype, control females and males, respectively. The offspring of F1 will be referred as F2.

After delivery of F2, social communication of F2 pups and maternal care of F1 females during the first postnatal week of F2 will be tested. See appendix 1 to this form, section A for further explanation of these outcomes as indicatives of an altered behavioural phenotype.

To test whether the depressive-like phenotype is expressed in F2, affective-related, cognitive, and social behaviours, gene expression and epigenetic markers of F2 will be assessed. Selected genes for mRNA expression analysis and measurement of epigenetic markers will be the same as for the study 1. See appendix 1 to this form, section A for detailed description of the general design of the animal procedures.

Milestone #1: Increased affective-related, or decreased cognitive or social behaviours between F2 born from depressed-like parents compared to F2 born from non-depressed-like parents.
Milestone #2: disturbed maternal care behaviour of depressed-like F1 female rats compared to non-depressed-like F1 female rats.
Milestone #3: Altered gene expression of selected genes of F2 born from depressed-like parents compared to F2 born from non-depressed-like parents.
Milestone #4: altered DNA methylation at the promotor regions of selected genes of F2 born from depressed-like parents compared to F2 born from non-depressed-like parents.

Milestone #5: similar DNA methylation profile at promotor regions of selected genes between depressed-like F1 and depressed-like F2.

Coherence: From a pilot study, we will investigate whether the combination of pregestational, prenatal and postnatal cumulative ELS induces a depressive-like behavioural phenotype (Phase 1). Next, we will explore whether this phenotype can also be reached with less early life stressors (Phase 2: study 1), and determine whether $SERT^{+/-}$ rats are more vulnerable than wildtypes (Phase 2: study 1). Also, we will explore the underlying molecular mechanisms of this depressive-like rat model (Phase 2: study 1). Follow-up studies will then zoom in on the social functioning in depression, its neurobiological mechanisms, and transgenerational effects (phase 2: studies 2 to 5).

3.4.2 Provide a justification for the strategy described above.

All studies proposed above are based on the principles of experimental research. For each study we have:

1. Established hypothesis to be empirically tested
2. Defined independent variables to be manipulated
3. Defined dependent variables to be measured
4. Followed experimental designs to establish relevant groups of comparison and control unknown and/or confounding variables

By following this strategy, we seek to establish a causal relationship between variables. We expect to demonstrate that the occurrence of depressive-like behaviours, altered gene expression and distinctive epigenetic markers of relevant genes (dependent variables) arise due to *cumulative* ELS exposure alone or in combination with the SERT genotype (independent variables).

The order in which the studies will be performed is important. In study 1 we will test if *cumulative* ELS induces a depressive-like phenotype in a robust fashion, alters gene expression and changes epigenetic signatures. We will only perform the following studies if a significant effect is found in this study. In studies 2 to 4 we will further characterise the social functioning and neurobiological mechanisms involved. Finally, we will investigate if the offspring (F2) of depressive-like parents (F1) also express a depressive-like phenotype, exhibit altered mRNA expression, and manifest changes in epigenetic markers.

By conducting these studies, we will answer the question whether the interaction of *cumulative* ELS and the SERT genotype increases the vulnerability to develop a depressive-like phenotype and provide mechanistic insights in stress-related disorders. In addition, we will understand how the neurodevelopmental trajectory of social functioning in depression, induced by ELS, is characterised. Finally, we will know to what extent the parental depressive-like phenotype is transferred to the next generation.

The selected strategy will allow us to optimise the use of animals and reduce their number where possible. The study 1 will be critical in this respect. Only the ELS treatment that produces the most robust depressive-like phenotype will be used in the follow-up studies. In addition, if no clear SERT genotype effect is found, the SERT$^{+/-}$ animal will not be used in the following studies.

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	Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.

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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 (0800-7890789).

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: Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter

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.

Phase 1
Pilot study
We will expose wildtype ($SERT^{+/+}$) and $SERT^{+/-}$ rats to *cumulative* ELS to test whether they develop depressive-like behaviours when they are adults.

For this research project *cumulative* ELS refers to the combination of (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS exposure. See the next section for detailed procedure of each type of ELS. $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to this *cumulative* ELS will be compared with $SERT^{+/+}$ and $SERT^{+/-}$ control animals.

From 14 weeks of life, $SERT^{+/+}$ and $SERT^{+/-}$ rats will be assessed for affective-related, cognitive and social behaviours. For this assessment, we will use the behavioural tests indicated below:

1- Elevated plus maze (EPM), sucrose preference test (SPT), and open field test (OFT) to evaluate affective-related behaviours.
2- Object location test (OLT) and novel object recognition (NOR) to test cognitive performance.
3- Social interaction test and social recognition test to assess social investigation, social memory and social withdrawal.

The tests will be conducted at least one week apart in the following order: EPM, SPT, OFT, OLT, NOR, social interaction test, and social recognition test. See next section about description of proposed animal procedures for detailed procedure of each behavioural test. After the last behavioural test (±20 weeks), animals will be sacrificed by fast decapitation and their brain tissue will be collected.

The chronology of this pilot study is summarised in fig1:

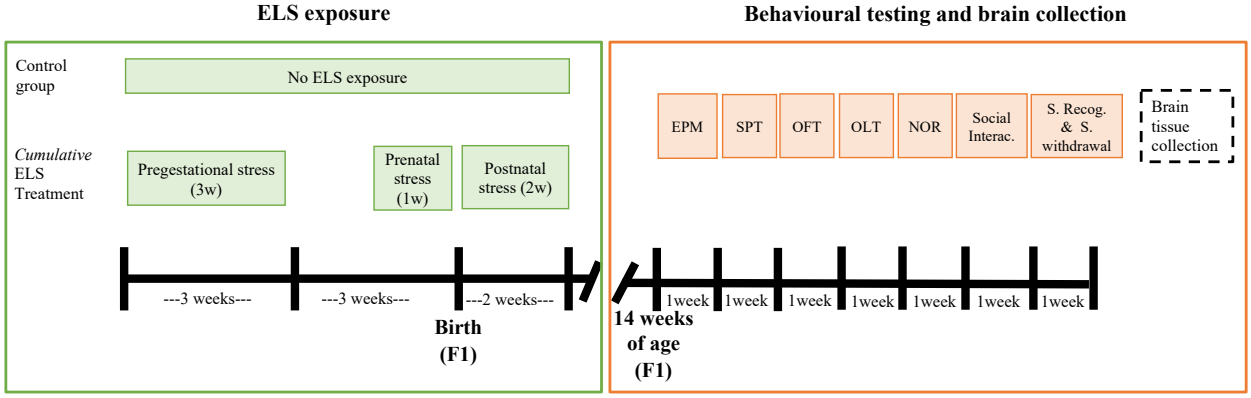

Fig1. General design of the pilot study.

-The EPM is useful test to evaluate anxiety-related behaviours. It is based on the natural tendency of rodents to avoid open spaces and stay in enclosed spaces. The more exploration in open arms, the more indication of reduced levels of anxiety.
-The SPT aims to evaluate the capacity of an animal to experience pleasure by consuming palatable food. It is based on the natural preference of rodents for sweets. Low levels of sucrose consumption are indicative of anhedonia, a central symptom in depressive disorders
-The OFT aims to evaluate anxiety-like behaviours. It is based on the natural tendency of rodents to avoid open spaces and exhibit thigmotaxis. The more time the animal spends walking along the walls or staying in the corners of the arena, the higher the index of anxiety-like responses.
-The OLT is useful to evaluate spatial short-term and long-term memory in rats. It is based on the spontaneous tendency of rodents, previously exposed to two identical objects, to later explore one of the objects—placed in a novel location—for a longer time than they explore the non-displaced object. The more time the animal spends with the object in the new location, the higher the level of spatial memory.
-The NOR aims to evaluate short and long-term recognition memory. It is based on rodents' natural tendency to explore novel features in their environment, including objects. The more time the animal spends with the new object, the higher the level of recognition
-Social interaction test, performed in an open arena, aims to evaluate investigation towards new social stimuli. It is based on the natural tendency of rodents to investigate unfamiliar mates. Several behaviours such as sniffing, grooming, or mounting can be measured as an index of social interaction.
-Social recognition test aims to assess the ability to recognise a novel mate in comparison to familiar mates. This test is based on the natural tendency of rodents to investigate unfamiliar partners. By performing this test in a three-chamber box, the more time spent in the box with the novel partner, the higher the index of social discrimination. An additional benefit of using this apparatus is the possibility to evaluate social withdrawal, i.e., the lack of motivation to have social contact. Social withdrawal can be assessed by measuring the time spent in the box in which neither the familiar nor the unfamiliar mate are present.

Two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to *cumulative* ELS.

This pilot study will follow a factorial experimental design with two factors: Factor 1: ELS treatment; Factor 2: SERT genotype.

The primary outcome parameters to test the onset of depressive-like behaviours induced by *cumulative* ELS will be the responses displayed by the rats in the behavioural tests mentioned above (also shown in orange boxes of fig1). The selection of these tests is based on previous research conducted in our research group and elsewhere [2-4] showing their validity to assess several parameters of affective-related behaviour, cognition, and social behaviours in rodents to obtain a comprehensive behavioral phenotype of the depressive-like rat model.

Phase 2

Study 1: the depressive-like phenotype by *cumulative* early life stress (ELS)

We will expose SERT$^{+/+}$ and SERT$^{+/-}$ rats to *cumulative* ELS to confirm whether they develop depressive-like behaviours in a robust fashion when they are adults. In contrast with the pilot study however, four conditions, one control and three experimental, will be compared in this study:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.
Experimental condition 1: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to postnatal ELS (ELS treatment 1).
Experimental condition 2: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to (maternal) prenatal ELS and postnatal ELS (ELS treatment 2).
Experimental condition 3 (*cumulative* ELS) : SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS (ELS treatment 3).

Similar to the pilot study, at 14 weeks of life rats will start to be behaviourally tested. See description of the pilot study above for further details about each behavioural paradigm and the order in which they will be used. After the last behavioural test (±20 weeks), animals will be sacrificed by fast decapitation and their brain tissue will be collected to analyse gene expression of selected genes, and epigenetic markers of those genes, in brain regions that are known to be involved in depression. Selected genes will at least include those involved in the serotonergic system, brain stress system (HPA axis), myelination, neural growth and survival as many of them can be co-expressed in major neural circuit connections involved in depressive-like behaviours in the rat (e.g., prefrontal cortex, hippocampus, amygdala) [1].

The chronology of this study is summarised in fig2:

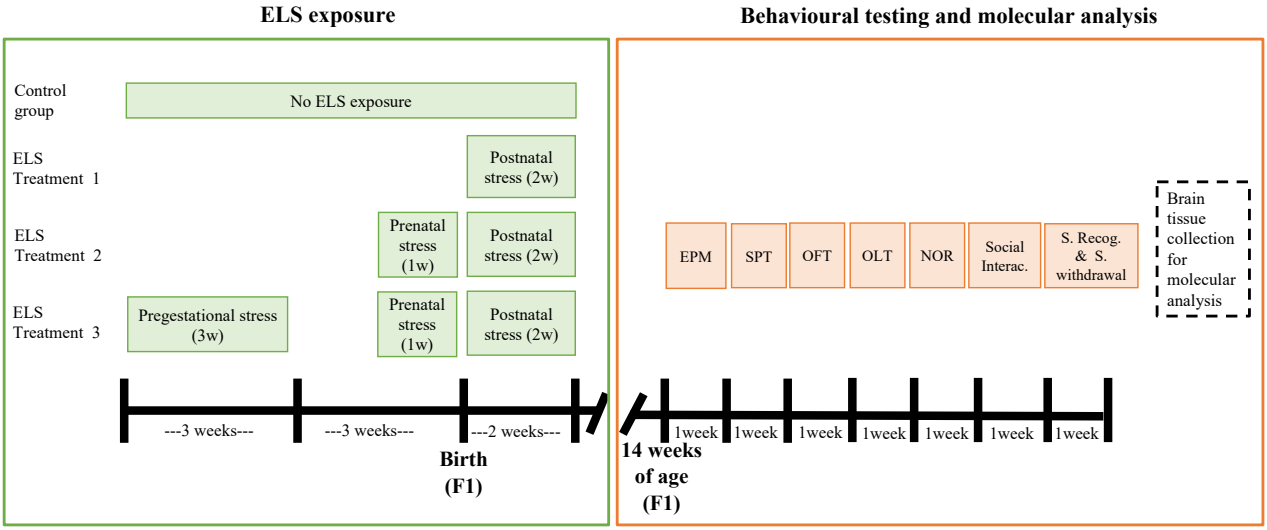

Fig2. General design of the study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: SERT genotype; Factor 3: Sex.

The primary outcome parameters to test the onset of depressive-like behaviours induced by *cumulative* ELS will be the responses displayed by the rats in the behavioural tests mentioned above (also shown in orange boxes of fig2). The selection of these tests is based on previous research conducted in our research group and elsewhere [2-4] showing their validity to assess several parameters of affective-related behaviour, cognition, and social behaviours in rodents.

The primary outcome parameter to test the changes of gene expression in the brain regions related to depressive-like behaviours induced by *cumulative* ELS will be the level of mRNA expressed for selected genes. The selection of mRNA gene expression analysis is based on previous evidence indicating that lower levels of mRNA expression of genes related to serotonergic system, brain stress system and neural growth correlated with maladaptive responses that increases the risk for depression [5]. Quantification of mRNA expression will be done by using the quantitative polymerase chain reaction (RT-qPCR) technique.

The primary outcome parameter to test epigenetic markers induced by *cumulative* ELS will be the level of DNA methylation at the promotor regions of selected genes. The DNA methylation analysis is selected because changes in DNA methylation (in general, hypermethylation) have been associated with depression [6].

Measurement of DNA methylation status in promotor regions of selected genes will be performed by PCR amplification of bisulfite-converted DNA [7].

It should be noted that additional ELS treatments without postnatal ELS are not relevant to this research project. If we exclude this period, the model will not resemble an important aspect of the human condition we seek to translate into the animal, i.e., adversity during the first years of life as a risk factor for depression later in life.

Studies 2 to 5 will be performed *only* if a depressive-like behavioural phenotype is found in this study. See section 3.4.1 of the project proposal form to see the explanation of selection points and decision criteria in this respect.

Study 2: focus on offspring social functioning in the juvenile period
Here we will test whether *cumulative* ELS alters social functioning in the juvenile period, and whether SERT$^{+/-}$ rats are more vulnerable compared with SERT$^{+/+}$ rats (depending on study1). To accomplish this, SERT$^{+/+}$ and SERT$^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

At ±4-5 weeks of age, animals will be assessed for social play behaviour in the social play-fighting test. This test will take place for 20 minutes (see next section for detailed procedure of social play-fighting test). After 90-120 minutes the social play-fighting test is over, animals will be sacrificed and brain tissue will be collected by perfusion to analyse the c-Fos protein expression level in brain regions that regulate social play behaviour (e.g., prefrontal cortex, dorsal and ventral striatum, amygdala, habenula) [8].

The chronology of this study is summarised in fig3:

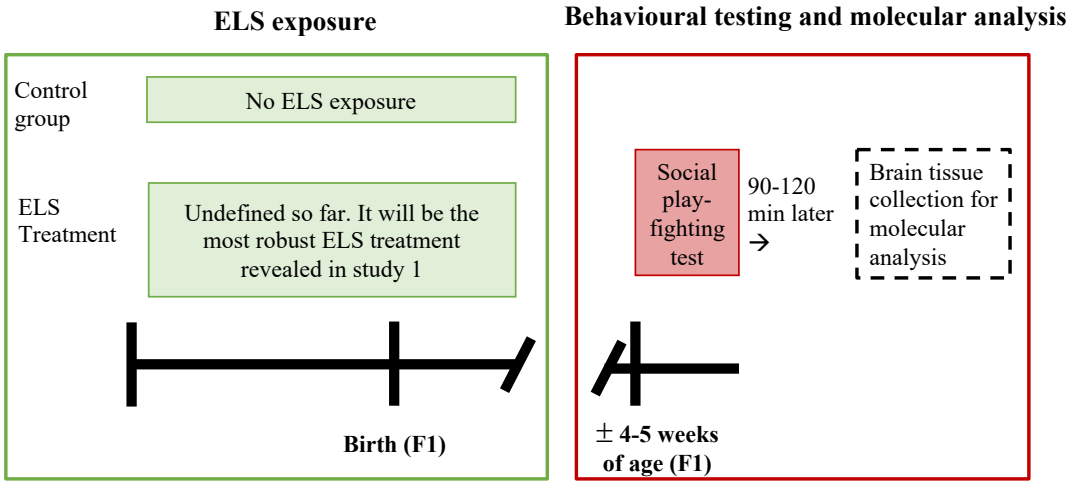

Fig3. General design of study 2

Two conditions, one control and one experimental, will be compared:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.
Experimental condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if SERT$^{+/-}$ animals are included).

The primary outcome parameters to test the changes in social play behaviour induced by *cumulative* ELS will be the responses displayed by the rats in the social play-fighting test. This test is selected because it allows to assess behavioural patterns of social play behaviour that are highly expressed in rodents at 35-42 days of age [9].

The primary outcome parameter to test changes in neuronal activity of brain regions that regulate social play behaviour induced by *cumulative* ELS will be the level of c-Fos protein expression in neurons of such brain regions. This expression level will be quantified through c-FOS protein immunohistochemistry staining to count c-Fos-positive neurons. c-Fos protein is expressed rapidly and transiently after external stimuli [10].

Therefore, the c-Fos protein expression level will serve as an indirect biomarker of neuronal activity in brain areas that regulate social play behaviour during the social play-fighting test.

Study 3: focus on offspring social functioning in adulthood
Here we will test whether *cumulative* ELS alters social functioning in adulthood, and whether SERT$^{+/-}$ rats are more vulnerable (depending on study1). To accomplish this, SERT$^{+/+}$ and SERT$^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

From 14 weeks of age, sexual behaviour of males and females will be tested once a week for 6-10 consecutive weeks. In order to prevent pregnancy in females during the sexual behaviour testing, a double tubal ligation surgery will be performed two weeks before the behavioural testing begins. See next section about description of proposed animal procedures of study 3, for details of this surgery procedure.

To test sexual behaviour in males, we will place the rat with a stimulus female in a testing cage. To test sexual behaviour in females, we will place the rat with a stimulus male in a testing cage. After the last sexual test has been taken place, males will be housed individually with a companion female in a new home cage to elicit territorial behaviour. Likewise, females will be housed individually in a new home cage to elicit territorial behaviour. One week later, we will use the resident-intruder test in males, and the female-intruder test in females to test aggressive behaviour. See next section about description of proposed animal procedures of study 3, for detailed procedure of each behavioural test.

As aggressive encounters induce a stress response in animals, we evaluate the stress response induced by the resident-intruder test in males and the female-intruder test in females. We will collect three blood samples from the animals' tail to assess the concentration level of corticosterone (the main stress hormone in rodents) in systemic circulation. We will collect three blood samples in total (one before, and two after the aggression test) because this will help us to track the changes in the corticosterone level as a result of being exposed to a stress challenge (i.e., the aggression test). See next section about description of proposed animal procedures of study 3, for detailed procedure of blood sampling.

After the resident-intruder test and collection of blood samples (90-120 minutes), animals will be sacrificed, and the brain tissue will be collected by perfusion to analyse the c-Fos expression level in brain regions that regulate aggressive behaviour [11].

The chronology of this study is summarised in fig4:

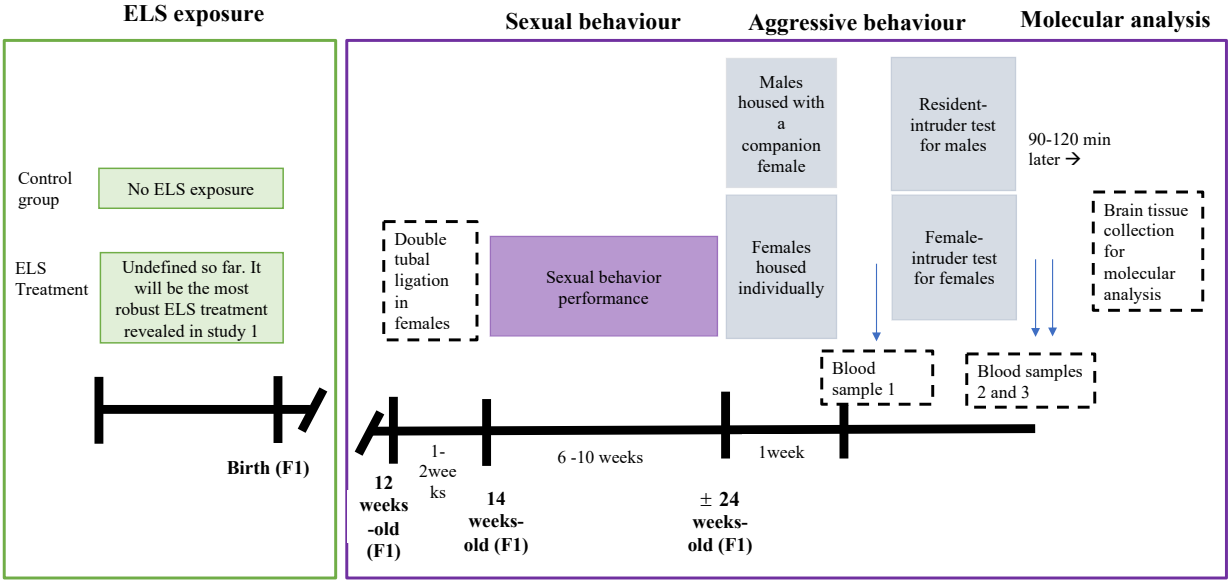

Fig4. General design of study 3. The symbol ± means approximately. It is possible that sexual behaviour testing takes fewer than 10 weeks. Therefore, the individual housing of males and females, the aggression test and the brain collection may take place few weeks earlier.

Two conditions, one control and one experimental, will be compared:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.

Experimental condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if SERT$^{+/-}$ animals are included).

Readouts of sexual behaviour test, resident-intruder test, and female-intruder test will be the primary outcome parameters to test the changes in adult social behaviour induced by *cumulative* ELS. Sexual behaviour testing is selected because adult males express copulatory behaviours in the presence of females, and females express proceptive and receptive behaviours (mediated by the oestrus cycle) in the presence of males. The resident-intruder test for males and the female-intruder test for females are selected because adult rodents express territorial aggression when encountering intruders in their own territory.

Systemic concentration level of corticosterone will be the primary outcome parameter to test whether *cumulative* ELS alters the stress response in adult rats following the exposure to a stress challenge (i.e., the aggression test). Blood sampling is selected because corticosterone is released from adrenal glands into blood circulation.

c-Fos-positive neurons expressed in brain regions that regulate aggressive behaviour will be the primary outcome parameter to test changes in neuronal activity induced by *cumulative* ELS. As mentioned above, c-Fos protein is expressed rapidly and transiently after external stimuli [10]. Therefore, the c-Fos protein expression level will serve as an indirect biomarker of neuronal activity in brain areas that regulate the aggressive behaviour during the aggression test.

Study 4: unravelling molecular mechanisms of social functioning
Here we will test the underlying molecular mechanisms related to the social brain network in adult animals exposed to *cumulative* ELS, and whether SERT$^{+/-}$ rats exhibit a different molecular pattern in comparison to SERT$^{+/+}$ (depending on study 1). To accomplish this, SERT$^{+/+}$ and SERT$^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

The animals will be left undisturbed (except for cage cleaning) until ±24 week of age in which they will be sacrificed by decapitation to collect the brain tissue. We will analyse the mRNA expression of selected genes in brain regions of the social brain network. We will also test the DNA methylation level at the promotors of those selected genes. Selected genes will at least include those that code for vasopressin, serotonin, corticoid and oxytocin receptors as many of them can be co-expressed in several regions of the social brain network.

The chronology of this study is summarised in fig5:

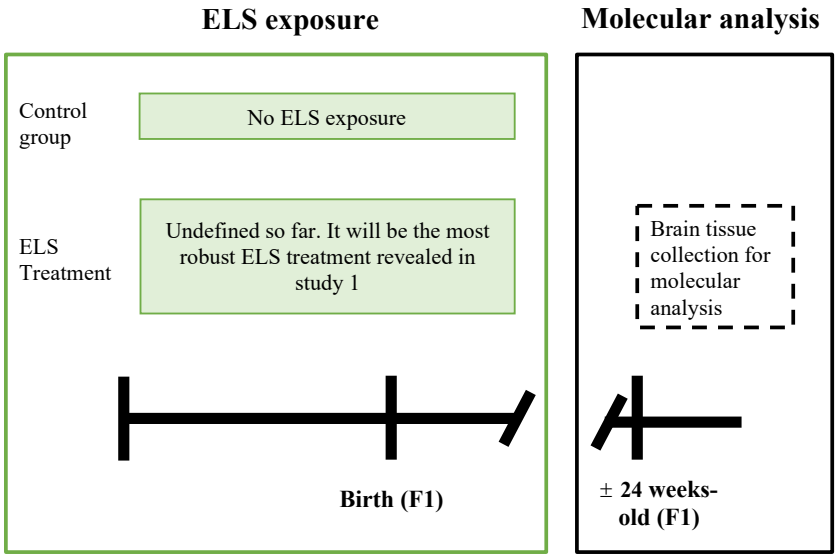

Fig5. General design of study 4. The symbol ± means approximately. This time point may vary depending on the time in which brain tissue will be collected in study 3.

Two conditions, one control and one experimental, will be compared:

Control condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats without any exposure to ELS.

Experimental condition: SERT$^{+/+}$ and SERT$^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

This study will follow a factorial experimental design with three factors: Factor 1: ELS treatment; Factor 2: sex; Factor 3: SERT genotype (only if SERT$^{+/-}$ animals are included).

No behavioural procedures will be performed before brain collection to prevent changes in gene expression and epigenetic markers as a result of behavioural tests. We will collect brains at ±24 week of age to make these results comparable to findings in study 3.

The primary outcome parameter to test the changes of gene expression in brain regions of the social brain network induced by *cumulative* ELS will be the level of mRNA expressed for selected genes. The selection of mRNA gene expression analysis is based on previous evidence indicating that gene expression of selected genes in brain regions comprising the social brain network are closely linked to expression of social behaviours like aggression [12]. Quantification of mRNA expression will be done by using the quantitative polymerase chain reaction (RT-qPCR) technique.

The primary outcome parameter to test epigenetic markers induced by *cumulative* ELS will be the level of DNA methylation at the promotor regions of selected genes in brain regions of the social brain network. The DNA methylation analysis is selected because changes in DNA methylation (in general, hypermethylation) have been associated with vulnerability to abnormal social functioning [13]. Measurement of DNA methylation status in promotor regions of selected genes will be performed by PCR amplification of bisulfite-converted DNA [7].

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
Here, we will determine if the depressive-like phenotype in generation F1 is transferable to their offspring F2. To accomplish this, first we will induce a depressive-like phenotype in F1 by exposing SERT$^{+/+}$ and SERT$^{+/-}$ rats to the ELS treatment that most robustly induced depressive-like behaviours in study 1. (SERT$^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, only SERT$^{+/+}$ rats exposed to ELS will be used).

When F1 males and females are adults, we will test affective-related behaviours to confirm that the depressive-like phenotype is expressed. To accomplish this, from 14 weeks of age (similar to study 1), affective-like behaviours of F1 will be tested by using the same tests used in study 1 (i.e., EPM, SPT and OFT). The tests will be conducted at least one week apart in the following order: EPM, SPT, OFT. After the last behavioural test, F1 animals will be mated to create F2. To do so, depressed-like males and depressed-like females will be mated with wildtype, control females and males, respectively.

F1 pregnant females will be left undisturbed during pregnancy (except for cage cleaning). After delivery of F2, we will measure social communication of F2 pups on postnatal day 6 and maternal care of F1 females during the first postnatal week of F2. See next section about description of proposed animal procedures of study 5, for detailed procedure of these behavioural tests.

When F2 are 14 weeks old (similar to study 1), we will test if the depressive-like phenotype is expressed. To do so, we will test affective-related, cognitive, and social behaviours of F2 as well as molecular mechanisms involved, by proceeding the same as we will proceed in study 1. Therefore, the same order and type of behavioural tests, as well as mRNA expression analysis and DNA methylation analysis will be conducted.

The chronology of this study is summarised in fig6:

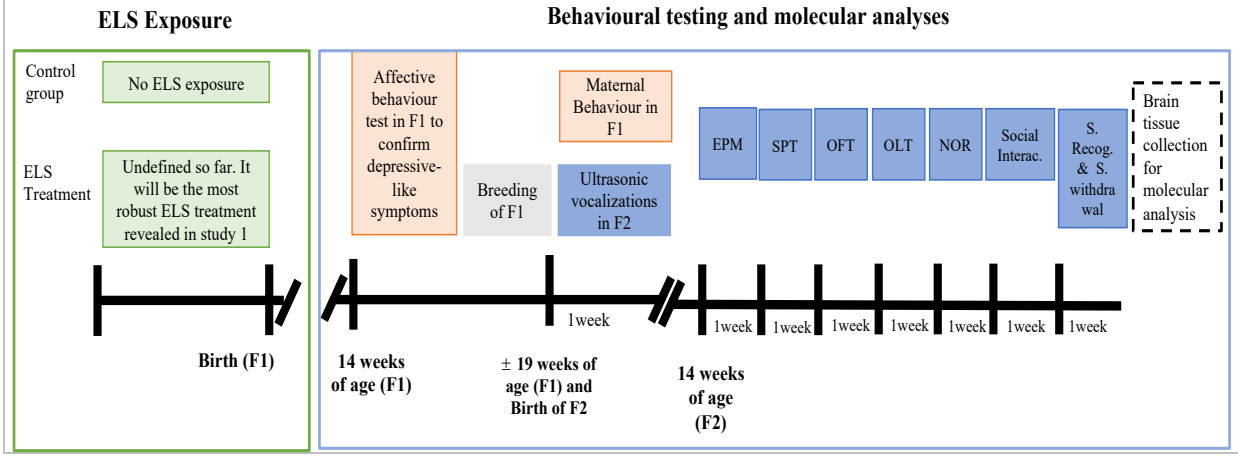

Fig6. General design of study 5. The symbol ± means approximately. Successful breeding of F1 may take longer. It is possible that depressed-like females will not get pregnant in the first attempt of breeding.

F1 and F2 outcomes will be analysed separately.

F1 analysis:
To analyse affective-related behaviours and maternal care of F1, two conditions, one control and one experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats without any exposure to ELS.
Experimental condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

The primary outcome parameter to confirm the depressive-like phenotype in F1 will be the responses that they will display in EPM, SPT and OFT.

The primary outcome parameter to test altered maternal care in F1 females will be the responses of care towards the pups expressed by the dam in the nest. Altered maternal care in humans is a clinical feature of postpartum depression (a type of depression); therefore, we will test whether the depressive-like phenotype in F1 females alters the maternal care.

F2 analysis:
To analyse altered social communication, depressive-like phenotype, gene expression, and epigenetic marks of F2, four conditions, one control and three experimental, will be compared:

Control condition: $SERT^{+/+}$ and $SERT^{+/-}$ rats born from $SERT^{+/-}$ F1 males mated with control $SERT^{+/+}$ F1 females.

Experimental condition 1: $SERT^{+/+}$ rats born from early-life stressed $SERT^{+/+}$ F1 males mated with control $SERT^{+/+}$ F1 females.

Experimental condition 2: $SERT^{+/+}$ rats born from early-life stressed $SERT^{+/+}$ F1 females mated with control $SERT^{+/+}$ F1 males.

Experimental condition 3: $SERT^{+/+}$ and $SERT^{+/-}$ rats born from early-life stressed $SERT^{+/-}$ F1 males mated with control $SERT^{+/+}$ F1 females.

Experimental condition 4: $SERT^{+/+}$ and $SERT^{+/-}$ rats born from early-life stressed $SERT^{+/-}$ F1 females mated with control $SERT^{+/+}$ F1 males.

The primary outcome parameter to test altered social communication in F2 pups induced by the F1 depressive-like phenotype will be the ultrasonic vocalizations emitted by the pups. The selection of this test is based on the natural ability of pups to transmit affective-related states to their mother through ultrasonic vocalizations. Reduced ultrasonic vocalizations of F2 will be an indicator of dysfunctional social communication very early in life as a result of being born from depressed-like parents.

The primary outcome parameters to test the F2 depressive-like phenotype induced by F1 parental-like depression will be the responses displayed by F2 rats in the behavioural tests indicated above (also shown in the blue boxes of fig6).

The primary outcome parameters to test changes in gene expression and epigenetic markers of F2 induced by F1 parental-like depression will be the level of mRNA expressed of selected genes and the level of DNA methylation at the promotor regions of selected genes in brain regions that are known to be involved in depression.

The selection of these behavioural tests and molecular analyses is based on evidence of offspring neurobiological and behavioural outcomes associated with stress/depression in the mother [14-16] and the father [17,18]. It is suggested that not only maternal stress can induce epigenetically driven effects on the offspring, but also paternal stress can induce long-lasting changes in germ cells, thus potentially inducing changes across generations [17].

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

Phase 1
Pilot study
SERT$^{+/+}$ and SERT$^{+/-}$ rats will be exposed to *cumulative* ELS. The procedures to induce (maternal) pregestational ELS, (maternal) prenatal ELS, and postnatal ELS are described below:

1) *pregestational ELS* refers to stressful events experienced by females before pregnancy to interfere with the development of the offspring. Pregestational stress will be applied to females for at least 21 days before pregnancy, induced by chronic unpredictable stress. We will follow the same protocol as [19,20]. Based on this protocol, females will be housed individually and subjected to 1–2 stressors per day for 3 weeks. Stressors will include restraint under bright light (1000 lux) for 1 h, overcrowding overnight (4 females per Macrolon type III cage), overnight exposure to damp bedding, 12h of food restriction, 5 minutes of forced swimming, and cage rotation for 12h. In accordance with the *Nationaal Comité advies dierproevenbelid* advice to prevent withdrawal of food for more than 24 hours in animals used for neurocognitive research [21], 12 hours of food restriction will be the maximum time used in this procedure and the female will be exposed to environmental enrichment; in addition, food restriction will not be applied in two consecutive days. Cage rotation will consist of changing the home cage location from one place to another from morning to afternoon, within the same experimental room. We select chronic unpredictable stress due to its previous validation to induce sustained changes in the stress response and depression-like responses in rodents. This procedure has been effective to induce neural changes and behavioural impairments in the offspring when it is applied to females before pregnancy. It was shown that pregestational stress caused lower viability of pregnancy, so about 1/3 of females did not became pregnant [22]. However, we will do some rebreeding attempts (max 3 attempts) to cover that.

2) *prenatal ELS* refers to stressful events experienced by females during their pregnancy to interfere with the development of the offspring. We will follow the same protocol as van den Hove et al. 2006 [23]. Females will be exposed to stress during the last week of pregnancy (14-21days), subjected to 3 sessions of 45-min restraint stress each day while being exposed to bright light. Dams will be put inside a plastic tube in which they can move their paws but cannot turn around. Each 45-min stress session will be as unpredictable as possible. We select restraint stress due to its efficacy to induce changes in the stress response and increase the likelihood of expressing depresssive-like behaviours. This procedure has been effective in inducing neural changes as well as affective-related and social impairments in the offspring when it is applied to females in the last week of pregnancy. Based on the van den Hove et al procedure, prenatal stress had no effect on gestational length, litter size, or pre-weaning mortality [23].

3) *postnatal ELS* refers to stressful events experienced during the first weeks of life (in rodents). In this research project, SERT$^{+/+}$ and SERT$^{+/-}$ rats will be exposed during the first two weeks of life (PND 2-15) to unpredictable maternal separation and disrupted maternal care induced by maternal stress. We will follow the same protocol as [4]. Unpredictable maternal separation in combination with maternal stress produces more persistent behavioural effects in the offspring. As a consequence of disrupted maternal care, the offspring is at higher risk of developing affective-related impairments later in life.

To induce unpredictable maternal separation, pups will be transferred as a whole litter into a new room for 3h/per day, starting at unpredictable time points each day. The whole litter will be placed in preheated Makrolon type II cages to prevent hypothermia (postnatal days 1-8: 32±1 °C; postnatal days 9-15: 28±1 °C). To disrupt maternal care behaviours, mothers will be exposed to maternal stress. Maternal stress will consist of either 20-min restraint stress or 5-minute forced swimming in cold water. They will be applied unpredictably and randomly during the separation of the litter. For restraint stress, dams will be put inside a plastic tube in which they can move their paws but cannot turn around. For the forced swimming, females will be placed into a cylindrical Plexiglas tank filled up with water at 18 °C.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. From 14 weeks of life, SERT$^{+/+}$ and SERT$^{+/-}$ rats (2 males and 2 females maximum per litter) will be behaviourally tested. General procedure for each behavioural test is indicated below:

1) EPM: The rat will be taken from the home cage and placed in the centre of the maze facing an open arm. The rat will be allowed to freely explore the maze for 5 min. Afterwards, the animal will be returned to the home cage.
2) SPT: Within his/her home cage, the rat will be exposed to one bottle of water and one bottle containing a sucrose solution for 24h on alternating days. On the other days two bottles of water will be presented. With each sucrose day, the sucrose concentration will be increased. Sucrose bottle locations on the cage will be alternated on sucrose days to prevent spatial bias. This test will take place for one week in total.
3) OFT: The rat will be taken from the home cage and placed in the centre of the open arena to freely explore the open field for 10 minutes. Afterwards, the animal will be returned to the home cage.
4) OLT: The rat will be taken from the home cage and placed into an open arena with two identical objects placed in two opposite corners. Free exploration will be allowed for 3 min (trial 1). After that, both the rat and the objects will be removed for 1 hr (to test short-term memory), after of which the next trial will start (trial 2). In this trial, the animal will be exposed to the same two objects as trial 1 for another 3 min; however, this time one of the objects will be place in a novel location. After the trial 2 is over, the animal will be returned to the home cage.
5) NOR: The procedure will be the same as for OLT. In this case, trial 1 will consist of exposure to two identical objects whereas trial 2 will consist of exposure to one familiar and one novel object placed in the same location during both trials.
6) Social interaction test: After the habituation to the arena, two rats that are unfamiliar to each other will be placed simultaneously in an open arena for 10 minutes. Each couple of animals will be matched by sex, SERT genotype, and ELS treatment. Afterwards, both animals will be returned to the home cage.
7) Social recognition test. After 10-minute habituation, the experimental rat will be allowed to explore an unfamiliar younger stimulus rat (stranger 1) that will be placed under a plastic grid in the left or right chamber of a three-chamber box, while the other chamber will contain an empty grid. After 10-minute exploration, a second unfamiliar younger stimulus rat (stranger 2) will be placed in the empty grid, and 10-minute exploration will be allowed. Afterwards, the animal will be returned to the home cage.

After all behavioural tests have been taken place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored for further molecular analyses.

Phase 2

Study 1: the depressive-like phenotype by *cumulative* ELS

$SERT^{+/+}$ and $SERT^{+/-}$ rats will be exposed to one, two, or three types of ELS depending on the experimental conditions they will be allocated to. See the animal procedures to induce (maternal) pregestational ELS, (maternal) prenatal ELS, and postnatal ELS described in the pilot study above.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. From 14 weeks of life, $SERT^{+/+}$ and $SERT^{+/-}$ rats (2 males and 2 females maximum per litter) will be behaviourally tested. General procedure for each behavioural test is the same as the one described in the pilot study above.

After all behavioural tests have been taken place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Study 2: focus on social functioning in the juvenile period

$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. At ±4-5 week of age, animals will be assessed for social play behaviour in the social play-fighting test. For this test, animals will be tested in couples. Each couple will be considered as an experimental unit and will consist of two unfamiliar mates matched by sex, SERT genotype, and ELS treatment. After the habituation to the testing cage (5 minutes), animals will be tested for 15 minutes. Boxing/wrestling, pouncing, pinning, chasing and social grooming will be scored.

After 90-120 minutes the social play-fighting test is over, the rat will be taken to a different room and will be anesthetised. Then, the rat will be sacrificed by perfusion and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Study 3: focus on social functioning in adulthood
$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. As of 14 weeks of age, sexual behaviour of males and females will be tested once a week for 6-10 consecutive weeks. To test sexual behaviour in males, we will place the rat with a stimulus female in a testing cage for 30 minutes. To test sexual behaviour in females, we will place the rat with a stimulus male in a testing cage for 30 minutes. Table 1 below indicates the sexual responses that will be scored in males and females. Immediately after the test, rats will be returned to their home cage.

After the last sexual test has been taken place, males will be housed individually with a companion female in a new home cage to elicit territorial behaviour. Likewise, females will be housed individually in a new home cage to elicit territorial behaviour. Water and food will be provided *ad libitum*. For home cage enrichment, wooden gnawing sticks, a polycarbonate small box for resting/sleeping and autoclaved bedding material will be provided. One week later, aggressive behaviour will be tested. For this assessment, we will use the resident-intruder test in males, and the female-intruder test in females.

The resident-intruder test will consist of the introduction of an unfamiliar, intruder male into the home cage of the resident male. The female-intruder test will consist of the introduction of an unfamiliar, intruder female into the home cage of the resident female. In both cases, the resident will correspond to the experimental animal, while the intruder will correspond to a same-sex, smaller stimulus rat that is unknown for the experimental animal. The aggression test will take place for 30 minutes and the behaviour of the resident will be recorded. Table 1 below indicates the aggressive responses to be scored in males and females. Immediately after the aggression test is over, rats will be placed in their home cage. From 90 to 120 minutes after the aggression test has been taken place, the rat will be taken to a different room and will be anesthetised. Then, the rat will be sacrificed by perfusion and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

Table 1. Readouts of sexual behaviour and aggressive behaviour

Sexual behaviour	**Aggression**
- Copulatory behaviour in males by scoring mounts, intromissions, and ejaculations - Receptive behaviour in females by scoring lordosis - Proceptive behaviour in females by scoring darts and hops and time spent with the male	- Attacks, lateral threat, upright posture, clinch attack, keep down and chase in males during the resident-intruder test. - Same parameters in females during the female-intruder test.

As aggressive encounters induce a stress response in animals, we will evaluate the stress response induced by the resident-intruder test in males and the female-intruder test in females. We will collect three blood samples from the animals' tail to assess the concentration level of corticosterone (the main stress hormone in rodents) in systemic circulation. Sampling will be made by making a small cut in the tail and collecting the blood from the dorsal tail vein. The first sample will be collected before the test to establish the baseline level of the hormone. The second sample will be collected immediately after the aggression test by removing the crust from the incision and collecting the blood. The third sample will be collected 30 minutes later by removing the crust (or making a new, small incision if needed) and collecting the blood. Each sampling will be maximum 300 uL, so the total amount of blood sampling will be < 1ml/kg. This procedure is considered as stress-free procedure and has been proven to be reliable to test corticosterone levels in systemic circulation in rodents [24].

Additional animal procedures in this study:
To prevent pregnancy in experimental females when the sexual behaviour is tested with the stimulus male, double tubal ligation surgery will be performed. This surgery will be also performed in stimulus females used to test sexual behaviour of experimental males. Likewise, same surgery will be performed in companion females

that will be caged with experimental males to conduct the resident-intruder test. In all three cases, the double tubal ligation surgery will be performed at least one week before the female is in contact with the male.

In preparation for the surgery, the rat will be anesthetised. To minimise discomfort directly linked to the surgery, analgesics will be subcutaneously injected at the start of the surgical procedure. The female will receive analgesics 24 hrs and 48 hrs hours after surgery. During the 7- 14-day recovery period, the weight and the well-being of the animals will be checked daily (on weekdays). Stimulus females used to test sexual behaviour of experimental males and companion females caged with experimental males for the resident-intruder test will be primed by subcutaneous injection of oestradiol to be behaviourally receptive while interacting with males.

Study 4: unravelling molecular mechanisms of social functioning
$SERT^{+/+}$ and $SERT^{+/-}$ (only if they are more vulnerable in study1) rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1.

After ELS exposure, rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype, and ELS treatment. The animals will be left undisturbed (except for cage cleaning) until ±24 week of age in which they will be sacrificed by rapid decapitation after CO_2 asphyxiation and brain tissue will be immediately collected and stored until the molecular analyses indicated in the previous section are performed.

Study 5: Transgenerational effects of the depressive-like phenotype by cumulative ELS
$SERT^{+/+}$ and $SERT^{+/-}$ F1 rats will be exposed to the ELS treatment that most robustly induced depressive-like behaviours in study 1. ($SERT^{+/-}$ rats will be used only if they are more vulnerable in study1; otherwise, $SERT^{+/+}$ rats will be used).

After ELS exposure, F1 rats will be weaned at postnatal day 21 and socially housed with mates matched by sex, SERT genotype and ELS treatment. From 14 weeks of age (similar to study 1), we will test F1 affective-related behaviours by using the same tests used in study 1 (i.e., EPM, SPT and OFT) and following the same procedure. After the last behavioural test, F1 animals will be mated. To do so, depressed-like males and depressed-like females will be mated with wildtype, control females and males, respectively, to produce F2.

F1 pregnant females will be left undisturbed during pregnancy (except for cage cleaning). After delivery of F2, we will measure social communication of F2 pups on postnatal day 6 and maternal care of F1 females during the first postnatal week of F2.

Social communication of F2 pups will be measured by ultrasonic vocalizations produced by the pup in response to separation from the dam and littermates in a 5-minute test. The pup will be individually transported from the nest to a testing room in which no other animals will be present. The pup will be place in a Makrolon type 2 cage filled with Aspen wood chip that will be under the ultrasonic microphone.

Maternal care of F1 females will be tested by scoring care behaviours displayed by the dam towards the pups in the nest, three times a day -30 minutes each- during the first postnatal week of F2. Licking/grooming, arched-back nursing and contact with pups will be scored in the home cage to minimise any disruption to the dams (F1) or the offspring (F2).

F2 rats will be weaned at postnatal day 21 and socially housed with mates matched by experimental condition (see previous section about description of general design for further explanation of control and experimental conditions). From 14 weeks of age (similar to study 1), we will test whether the depressive-like phenotype is expressed in F2. Therefore, we will test F2 affective-related, cognitive, and social behaviours and molecular mechanisms involved by proceeding the same as we will proceed in study 1. After all behavioural tests have been taking place, the animal will be taken to a different room and will be exposed to CO_2 asphyxiation. Immediately after, the rat will be sacrificed by rapid decapitation and the brain tissue will be collected and stored until the molecular analyses indicated in the previous section are performed.

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

To estimate the sample size of each study, we used the Gpower 3.1 statistical software. The input parameters to calculate the sample size of each study for ANOVA (fixed effects, special, main effects, and interactions) statistical tests depended on three aspects: 1) the number of groups per study; 2) assumptions of power and alpha for statistical significance and, 3) the effect sizes reported in previous studies.

Aspect one: The number of groups required per study:

- Pilot study = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 1 = 16 groups (4 treatments, 2 SERT genotypes, 2 sexes)
- Study 2 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 3 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)
- Study 4 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes)

As in study 5 two generation of rats will be tested (i.e., F1 and F2), the estimation of animals for this study is as follow:

- F1 = 8 groups (2 treatments, 2 SERT genotypes, 2 sexes).
- F2 = 16 groups (4 parental conditions, 2 SERT genotypes, 2 sexes). See previous section about description of general design, study 5, for further explanation of control and experimental conditions of F2.

Aspect two: We based our calculations on the assumption to obtain a significant group effect with a power of 80% and alpha value of 0.05.

Aspect three:

For the pilot study, we used the effect size f=0,32 calculated from the finding of reduced sucrose preference induced by the ELS treatment reported by 5.1 lid2e, 5.1 lid2h [4], chapter 4. We chose this primary outcome because it is indicative of anhedonia, a key symptom in depression.

For behavioural studies 1, 2, 3, and 5, we used the effect sizes calculated from behavioural results reported by 5.1 lid2e, 5.1 lid2h [4], chapter 8. Specifically:

For studies 1 and 5, the effect size f=0,227 was calculated from the finding of reduced sucrose preference induced by the ELS treatment. We chose this primary outcome because it is indicative of anhedonia, a key symptom in depression.

For studies 2 and 3, the effect size f=0.253 was calculated from the finding of lower sociability induced by the ELS treatment. We chose this primary outcome because it is indicative of social withdrawal, also reported in depression.

Even though we do not know the behavioural outcomes of our proposed studies, especially of animals exposed to the cumulative ELS, we consider the effect sizes from findings reported by 5.1 lid2e, 5.1 lid2h as appropriate for our estimations as they also studied the interaction of ELS and the SERT genotype to test animals' behaviour and used several of the same tests we have proposed here.

For study 4, we used the effect size based on the findings reported by 5.1 lid2e, 5.1 lid2h [25]. Specifically, we used the effect size f=0,337 calculated from the finding of upregulated myelin-related gene expression in prefrontal cortex and downregulated myelin-related gene expression in basolateral amygdala of male rats exposed to ELS treatment. We chose this primary outcome because it indicates an effect of ELS exposure on molecular mechanisms of neural circuit connections involved in depressive-like behaviours.

Even though we do not know the molecular outcomes of animals exposed to ELS in our proposed study, we consider the effect size from findings reported by 5.1 lid2e, 5.1 lid2h appropriate for our estimations as they also studied the exposure to ELS as one factor influencing the gene expression and epigenetic regulation of some of the same brain regions we have proposed to analyse here.

Other considerations to calculate the number of animals:

1- Animals to be tested in all studies are born from mothers exposed to stress, therefore, we also estimated the number of females to be exposed to stress. This calculation is described in section B of this document, number of animals.
2- Animals to be used in studies 2 to 4 will be born from the same mothers. Therefore, we will assign 1 male and 1 female per litter to each study. On one hand, this will maximise the use of the offspring; on

the other, it will control litter effects for molecular analysis involved in studies 2 to 4. Even though we do not know the behavioural outcomes of studies 2 and 3, the brains for study 4 will be collected to optimise animal use (use of the same litters from studies 2 and 3).

Minimising the number of animals in studies 2 to 5 might be possible based on outcomes of study 1. If results from this study indicate that $SERT^{+/-}$ rats are not more vulnerable than $SERT^{+/+}$ animals, we will not use this knockout manipulation for studies 2 to 5; therefore, the number of animals needed for those studies might be reduced.

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
A2	Rattus norvegicus	Animals bred in our animal facility	Adult females to expose to stress and breed. Newborn, juvenile, and adult offspring to test behaviour and for molecular analysis	3382	Both	Yes	Wistar

Provide justifications for these choices

Species	Affective-related, cognitive and social behaviours in the rat resemble functional similarities of the same behaviours in humans. Similar to humans, rats are also highly sensitive to stress exposure very early in development. Changes in rat behaviour following *cumulative* ELS can resemble human behavioural changes after a long history of ELS. In addition, the brain circuit for the expression of affective-related, cognitive and social behaviours is highly conserved across mammals. Therefore, brain circuit functionality in the rat resembles brain circuit functionality in humans.

Origin	**Phase 1** **Pilot study:** F0 = SERT$^{+/-}$ males mated with SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0 **Phase 2** **Study 1:** F0 = SERT$^{+/-}$ males mated with SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0 **Studies 2 to 4:** F0 = SERT$^{+/-}$ males mated with SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups. This generation will be the parents of rats to be used in studies 2 to 4 F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0 **Study 5:** Origin of animals for control condition: F0 = SERT$^{+/-}$ males mated with control SERT$^{+/+}$ females. SERT$^{+/+}$ females will be selected to control maternal behaviour when nurturing F1 pups F1 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F0, and mated with control SERT$^{+/+}$ rats to created F2 F2 = SERT$^{+/+}$ and SERT$^{+/-}$ rats born from F1 Origin of animals for experimental conditions 1 and 2: F0 = SERT$^{+/+}$ males mated with stressed SERT$^{+/+}$ females F1 = stressed SERT$^{+/+}$ rats born from F0, and mated with control SERT$^{+/+}$ rats to create F2 F2 = SERT$^{+/+}$ rats born from stressed SERT$^{+/+}$ F1 rats Origin of animals for experimental conditions 3 and 4: F0 = SERT$^{+/-}$ males mated with stressed SERT$^{+/+}$ females F1 = stressed SERT$^{+/-}$ rats born from F0, and mated with control SERT$^{+/+}$ rats to create F2 F2 = SERT$^{+/+}$ rats and SERT$^{+/-}$ rats born from stressed SERT$^{+/-}$ F1 rats
Life stages	**Phase 1** **Pilot study** *Adult females:* for stress exposure and breeding at 3 months of age *Adult offspring:* for testing the depressive-like phenotype **Phase 2** **Study 1** *Adult females:* for stress exposure and breeding at 3 months of age *Adult offspring:* for testing the depressive-like phenotype and molecular analysis of brain tissue **Studies 2 to 4** *Adult females:* for stress exposure and breeding at 3 months of age *Juvenile offspring to study 2:* for social play testing and molecular analysis of brain tissue *Adult offspring to study 3:* for sexual behaviour and aggression testing and molecular analysis of brain tissue *Adult offspring to study 4:* for molecular analysis of brain tissue at the same age of rats used in study 3 to make outcomes of molecular analysis comparable **Study 5** *Adult females* (F0): for stress exposure and breeding at 3 months of age *Adult offspring* (F1): for testing the depressive-like phenotype and breeding *Newborns and adult offspring* (F2): for testing transgenerational behavioural effects of parental depressive-like phenotype and molecular analysis of brain tissue

Number

Phase 1

Pilot study

We will need n=10 animals per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 10 x 8 = 80. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed is 80 / 4 = 20. We estimated 10 pups delivered by each F0 female, so 20 x 10 = 200. If we use 2 male and 2 female pups per litter to get the total sample of 80 rats, 6 pups per litter will not be used (20 X 6 = 120). See calculations per treatment as follows:

F0 females exposed to ELS N=20		F1 rats used (2 males + 2 females per litter) N= 80		F1 rats exposed to ELS non-used for pilot study (6 pups per litter) N= 120	
Controls	Cumulative ELS	Controls	Cumulative ELS	Controls	Cumulative ELS
10	10	40	40	60	60

Exposure to stress in F0 females can interfere with normal body weight, affect their welfare, reduce the rate of fertilization, or alter pregnancy maintenance. Therefore, we estimated extra F0 females to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the cumulative ELS groups, we based our calculations according to the 1/3 of non-pregnancy rate reported by Gemmel et al [22]; hence we estimated 30% extra for cumulative ELS. In addition, extra F1 pups born from extra F0 females were also estimated to be used because stressed mothers might deliver fewer than 10 pups per litter, or animals born from stressed mothers might not survive due to impaired maternal care. See calculations of all extra rats per treatment as follows:

Extra F0 females for possible dropouts N=3		Extra F1 rats for possible dropouts (2 males + 2 females per litter) N=12		Extra F1 rats exposed to ELS non-used for pilot study (6 pups per litter) N=18	
Controls	Cumulative ELS	Controls	Cumulative ELS	Controls	Cumulative ELS
0	3	0	12	0	18

Phase 2

Study 1:

We will need n=14 animals per group; and we will have 16 groups (4 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 14 x 16 = 224. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed 224 / 4 = 56. We estimated 10 pups delivered by each F0 female, so 56 x 10 = 560. If we will use 2 male and 2 female pups per litter to get the total sample of 224 rats, 6 pups per litter will not be used (56 X 6 = 336). See calculations per treatment as follows:

F0 females exposed to ELS N=56				F1 rats used (2 males + 2 females per litter) N= 224				F1 rats exposed to ELS non-used for study 1 (6 pups per litter) N= 336			
Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3
14	14	14	14	56	56	56	56	84	84	84	84

Abbreviations: ELS-T1 = Postnatal stressors; ELS-T2 = Prenatal and postnatal stressors; ELS-T3 = Pregestational, prenatal and postnatal stressors.

Exposure to stress in F0 females can interfere with normal body weight, affect their welfare, reduce the rate of fertilization, or alter pregnancy maintenance, especially in females exposed to ELS-T3. Therefore, we estimated extra F0 females to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations according to the 1/3 of non-pregnancy rate reported by Gemmel et al [22]; hence we estimated 30% extra for ELS-T3. We estimated 15% extra for ELS-T2 and ELS-T1 each based on possible (although unlikely) reduced gestational length, litter size, or pre-weaning mortality [23]. In

addition, extra F1 pups born from extra F0 females were also estimated to be used because stressed mothers might deliver fewer than 10 pups per litter, or animals born from stressed mothers might not survive due to impaired maternal care (especially ELS-T3. See calculations of all extra rats per treatment as follows:

Extra F0 females for possible dropouts N=11				Extra F1 rats for possible dropouts (2 males + 2 females per litter) N= 44				Extra F1 rats exposed to ELS non-used for study 1 (6 pups per litter) N= 66			
Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3	Controls	ELS-T1	ELS-T2	ELS-T3
0	3	3	5	0	12	12	20	0	18	18	30

Studies 2 to 4:
For study 2, we will need n=16 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 16 x 8 = 128.

For study 3, we will need n=16 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 16 x 8 = 128.

For study 4, we will need n=9 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 9 x 8 = 72.

Animals needed for studies 2 to 4 will come from the same litter and be born from dams (F0) exposed to stress. Therefore, the total number of F0 females needed is = 64. We estimated 10 pups delivered by each F0 female, so 64 x 10 = 640. If we will use 128 rats to the study 2, 128 rats to the study 3, and 72 rats to the study 4 (to get a total of 328 rats), the total of non-used animals will be =312. See calculations per treatment as follows:

	F0 females exposed to ELS N= 64		F1 rats used N=328		F1 rats exposed to ELS non-used for studies 2 to 4 N=312	
	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
Study 2	32	32	64	64	156	156
Study 3			64	64		
Study 4			36	36		

As in study 3 surgery for tubal ligation will be performed in females, we also estimated extra females to be used for possible dropouts related to this procedure. Therefore, if we will have 4 groups of females (2 treatments, 2 SERT genotypes), we estimated one extra female per group, therefore, n of dropouts=4

We also estimated extra F0 females and extra F1 pups to be used for possible dropouts for same reasons indicated above. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations assuming that the most robust ELS will be the ELS-T3; hence we estimated 30% extra. See calculations of all extra rats as follows:

	Extra F0 females for possible dropouts N= 10		Extra F1 rats for possible dropouts N=52		Extra F1 rats exposed to ELS non-used for studies 2 to 4 N=48	
	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
Study 2	0	10	0	20	0	48
Study 3			0	20		
Study 4			0	12		

The estimated number of companion females to test males' sexual behaviour is = 64
The estimated number of companion females to be housed with males for aggression test is = 64

Study 5:

Estimated number of F1 rats

We will need n=20 rats per group; and we will have 8 groups (2 treatments, 2 SERT genotypes, 2 sexes); therefore, total sample size is 20 x 8 = 160. Rats to be tested will be born from dams (F0) exposed to stress. As we will use 2 male and 2 female pups born from each F0 female, total number of F0 females needed is 160 / 4 = 40. We estimated 10 pups delivered by each F0 female, so 40 x 10 = 400. If we will use 2 male and 2 female pups per litter to get the total sample of 160 rats, 6 pups per litter will not be used (40 X 6 = 240). See calculations as follows:

F0 females exposed to ELS N=40		F1 rats for behavioural testing and breeding N=70		F1 rats for behavioural testing only N=90		F1 rats exposed to ELS non-used for study 5 N=240	
Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
20	20	14	56	66	24	120	120

We also estimated extra F0 females and extra F1 pups to be used for possible dropouts. Even though we do not know the exact percentage of dropouts of the ELS groups, we based our calculations assuming that the most robust ELS will be the ELS-T3; hence we estimated 30% extra. See calculations of all extra rats as follows:

Extra F0 females for possible dropouts N=6		Extra F1 rats for possible dropouts (2 males + 2 females per litter) N=24		Extra F1 rats exposed to ELS non-used for study 5 (6 pups per litter) N= 36	
Controls	Most robust ELS	Controls	Most robust ELS	Controls	Most robust ELS
0	6	0	24	0	36

Estimated number of F2 rats

We will need n= 14 rats per group; and we will have 16 groups (4 parental conditions, 2 SERT genotypes, 2 sexes); therefore, total sample size is 14 x 16 = 224. The parents of these animals will be F1 rats used for testing and breeding, indicated in the table above (N=70). We estimated 10 pups delivered by each F1 parent, so 70 x 10 = 700. If we will use 56 control animals and 168 experimental animals to get the total sample of 224 rats, n=476 will not be used. See all calculations below:

F2 rats used for behavioural testing and brain collection N=224		F2 rats born from F1 non-used for study 5 N= 476	
Controls	Experimental groups	Controls	Experimental groups
56	168	84	392

We also estimated extra F2 animals born from extra F1 rats because animals born from F1 depressed-like mothers might not survive due to impaired maternal care, or litters from SERT$^{+/-}$ parents might be smaller. See calculations as follows:

Extra F2 rats born from extra F1 animals N=96		Extra F2 born from extra F1 non-used for study 5 N= 144	
Controls	Experimental groups	Controls	Experimental groups
0	96	0	144

Sex	Depression in humans is expressed in both sexes, therefore we will conduct this research in male and female rats.
Genetic alterations	$SERT^{+/-}$ rats that express lifetime low expression of the serotonin transporter (SERT) will resemble low SERT expression in human carriers of the short allele in the SERT gene.
Strain	Wistar

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, except for females exposed to stress before and/or during pregnancy

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

Females exposed to stress before pregnancy (pregestational ELS) will be individually housed to be exposed to chronic unpredictable stress. During this procedure, females will be housed with enough bed material, gnawing sticks, and a polycarbonate small box for resting/sleeping for environmental enrichment to cope with stress. Females will have free access to food during the whole procedure, except when food restriction is induced. In accordance with the *Nationaal Comité advies dierproevenbelid* advice to prevent withdrawal of food for more than 24 hours in animals used for neurocogntive research [21], 12 hours of food restriction will be the maximum time used in this procedure and it will not be applied in two consecutive days. Water will be provided *adlibitum* during the whole procedure.

Females exposed to stress during pregnancy (prenatal ELS) will be housed two animals per cage during the first two weeks of pregnancy. During the third week of pregnancy, females will be individually housed to be exposed to stress and for delivery. During individual housing, pregnant dams will be housed with enough bed material, gnawing sticks, and a polycarbonate small box for resting/sleeping for environmental enrichment to cope with stress, and they will have free access to water and food during the whole procedure.

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.

- In study 3, one or two tail incisions (in case taking off the crust from the first incision does not work) will be made to collect the blood as described in section A of this form. Pain relieving will not be used for this procedure because the severity is mild and, if properly executed, it is not expected to cause any detectable adverse effect.
- Studies 1 and 4, and pilot study involve brain tissue collection after decapitation to perform molecular analysis. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible. Anaesthesia is not recommended before decapitation because it can alter the analysis of gene expression.

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

- Studies 2 and 3 involve brain tissue collection after perfusion to perform analysis of c-Fos expression. Preparation for perfusion will involve an intraperitoneal overdose of pentobarbital for deep anaesthesia and non-recovery. The dose will be adjusted for each animal's weight.
- For double tubal ligation to females in study 3, each female will be under anaesthesia. To minimise discomfort directly linked to the surgery, analgesics will be subcutaneously injected. The female will receive analgesics 24 hrs and 48 hrs hours after surgery. During the 14-day recovery period, the weight and the well-being of the animals will be daily checked.

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

- Chronic unpredictable stress applied to females before conception may reduce their body weight and/or interfere with fertilization.
- Restraint stress in pregnant females may affect their body weight, the maintenance of pregnancy, the number of offspring alive after delivery and/or litter size at birth.
- Unpredictable maternal separation and disrupted maternal care may potentially increase pups' mortality and/or may reduce the body weight of both mothers and the litter.
- Isolation of females 1 week prior to female-resident intruder test may induce stress (study 3)

Explain why these effects may emerge.

- Prolonged exposure to stress dysregulates the release of glucocorticoids. This interferes with the normal activity of glucose, thus affecting the metabolism of animals. In addition, high levels of glucocorticoids induced by stress can interfere with normal functioning of sexual hormones associated with embryo implantation.
- Exposure to high levels of stress during pregnancy increases the levels of glucocorticoids into systemic circulation that can interfere with the functioning of the placenta or foetal development. This situation can alter the maintenance of gestation, normal foetal growth, or number of pups alive after delivery.
- Separation from mothers or disrupted maternal care may reduce the amount or quality of mothers-litter nurturing, therefore, these procedures may reduce the amount or quality of nesting, lactation, or mothers' licking behaviour towards their litters, all of which are necessary for pups' development.
- Social isolation is stressful for social animals.

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

- We will do 3 attempts of rebreeding (maximum) as soon as the pregestational stress procedure is over. Pregestational stress procedure can finish 0-2 weeks before the gestational period begins without interfering with the experimental outcome [26].
- We will provide sufficient food and water ad libitum to maintain body weight as normal as possible.
- We will provide enough bed material, gnawing sticks and group housing for environmental enrichment that helps to cope with stress.
- We will provide food and water ad libitum, enough bed material and gnawing sticks for environmental enrichment for isolated females in study 3.
- We will monitor that animals are not exposed to sources of stress other than stressors used for the experiments. This means we will prevent a noisy environment, inadequate room temperature, light, or ventilation, inadequate handling, etc.

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.

If animals show clear signs of the sickness behaviour and/or display grimace signs of pain (arched back, rough coat, general malaisse) or loose more than 15% of their body weight in 48 hours, they will be excluded from the experiment and terminated. If they show tumors, they will be terminated. We will not interfere with the pups until they are weaned (that is, outside the maternal separation, we will leave the nest with the mom).

Indicate the likely incidence.

Very low. Although stress procedures are chronic, our previous experience as well as research conducted elsewhere have shown that the level of discomfort induced by selected stress procedures do not significantly interfere with feeding, water consumption, motor activity, or cleaning behaviours. Based on van den Hove et al [23] procedure, prenatal stress had no effect on gestational length, litter size, or pre-weaning mortality, so restraint stress effects on pregnant rats are very unlikely. As far as we know, nobody has performed studies combining pregestational, prenatal and postnatal ELS, therefore, we are not sure about the incidence of negative effects. With drug treatments (maternal fluoxetine use) we used before we saw viability index of 72-73%. We expect these stressors to have a higher viability index as we saw that only one ELS had no significant effects [Chapter 5 in ref 4]. If 28% would die, we still have enough animals left in the litter. We will monitor the welfare of the females and offspring to properly record it and take actions to implement humane endpoints if applicable.

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).

For the pilot study and the following studies if the stress procedure works to induce the depressive-like phenotype, we expect the pregestational, prenatal and postnatal stressors to be in the range of moderate. Especially because in our hands, the pre- and postnatal stressors have never led to a higher discomfort than 'moderate'. The combination of all three stressors is new, and we will monitor the animals closely and will contact immediately the Institutional Animal Care and Use Committee when the discomfort will be higher than anticipated (or when in doubt). Experiments will end (in consultation with the IACUC) when they exceed "moderate" discomfort levels. The animal behavior tests that the animals will undergo, are similar to what we have performed before in our lab and are in the range of mild. Cumulative discomfort for this group will therefore be estimated as 'moderate'.

For all other groups, the behavioral tests we will use are tests we have ample experience with. In our hands, they never exceeded 'moderate' discomfort levels. The early life stressor with the most robust effects will be used, which may mean the pregestational, prenatal and postnatal stressors might be picked as the preferred model. We expect the discomfort of the mother to have an impact on the offspring, however we still expect this to be in the 'moderate' range when combined with the behavioral experiments (which are mild to moderate).

Of a total of estimated animals to be used, it is expected that 22,5% of animals are exposed to cumulative mild discomfort and 77,5% are exposed to cumulative moderate discomfort.

	All studies		Pilot study & Study 1		Study 2		Study 3		Study 3		Study 4		Study 5		Study 5		Companion females housed with males + stimulus females to test males' sexual behavior in study 3	All studies		Study 5	
	F0		F1		F1		F1 males		F1 females		F1		F1		F2			Non-used F1 rats		Non-used F2 rats	
	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	ELS	CTR	EXP		CTR	ELS	CTR	EXP
Arrival	2	2																			
Handling	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2				
Stress exposure		3		3		3		3		3		3		3					3		
Females housing for stress exposure before & during pregnancy		3																			
Cognitive tests			2	2											2	2					
Affective tests			2	2									2	2	2	2					
Social tests			2	2	2	2	3	3	3	3					2	2					
Home accommodation to induce territorial behaviour							2	2	3	3											
Repeated blood sampling							3	3	3	3											
Tubal ligation surgery									3	3							3				
Primed s.c. oestradiol treatment (max 1x every two weeks)																	2				
Euthanasia	1	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
Cumulative discomfort (SV)	SV2	SV3	SV3	SV3	SV2	SV3	SV3	SV3	SV3	SV3	SV2	SV3	SV2	SV3	SV3	SV3	SV3	SV2	SV3	SV2	SV3
% of animals to have expected cumulative discomfort	2,2	4,0	2,8	7,8	1,9	2,5	1,0	1,2	1,0	1,3	1,1	1,4	2,4	3,1	1,7	7,8	3,8	12,4	22,3	2,5	15,8

***** *EU directive scale: SV1=terminal, SV2=mild, SV3=moderate, SV4=severe*

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	The measurement of the depressive-like phenotype in the rat model implies the assessment of complex patterns of behaviours to resemble the human condition. *In vivo* studies

	conducted with rats allow to investigate a wide range of behaviours that are functionally similar to those observed in humans, as well as the underlying mechanisms in the brain. Additionally, the functioning of brain regions involved in regulation of affective-related, cognitive, and social behaviours in humans is highly conserved in rats and cannot be found in lower organisms; therefore, the use of these types of live animal experiments is still irreplaceable. Moreover, *in vitro* studies on isolated tissue would not be able to give a sufficient overview of the effect of *cumulative* ELS in the brain and behaviour in the offspring. In short, it is not feasible to replace the use of live animals in this research project.
Reduction	To assess the effects of cumulative ELS and SERT gene interaction, intact and freely behaving rats will have to be used. We have developed in-depth expertise in the proposed animal experimental paradigms; in addition, we have selected validated, optimised, and refined protocols that will reduce the number of animals needed to obtain significant results. The number of animals needed for these studies will be carefully considered based on prior studies and expected variance in the dependent variables. Furthermore, we consider carefully which models are needed for our studies. First, the go/no go moments are described in the section 3.4.1 of the proposal form. If findings from the pilot study do not show behavioural primary outcomes of a depressive-like phenotype in rats exposed to cumulative ELS, conducting phase 2 will no longer be relevant. Furthermore, if $SERT^{+/-}$ rats do not express higher vulnerability to depressive-like behaviours than $SERT^{+/+}$ rats, they will no longer be used in studies of phase 2. Hence, only $SERT^{+/+}$ rats will be included and the number of groups and animals in studies 1 to 5 would be reduced. Second, estimated number of animals per group per study is based on statistical methods aimed at minimising the number of animals. Third, the offspring of studies 2 to 4 will come from the same parents, thus maximising the use of the offspring.
Refinement	All procedures are used regularly in our laboratory and have been previously refined to minimise the potential discomfort. The responsible researchers have ample expertise with these procedures and with training other researchers. The animal housing facilities are well-equipped to house rodents. The responsible researchers and the staff from the animal facilities are properly trained to handle the animals; assess the health and welfare of the animals; administer anaesthesia and minimise pain and suffering. All surgical procedures will be performed under anaesthesia, with proper post-operative care. Animals will be humanely killed at defined end points according to national ethical rules. Stress procedures will produce discomfort in animals. However, ad libitum food and water (unless stated otherwise) and environmental enrichment through social housing, gnawing sticks, and sufficient nesting material in the home cage in all studies will be ensured to increase strategies to cope with stress.

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.

In study 5, non-stressed F1 rats can be re-used for educational purposes as discomfort in them is classified as mild. However, if they are declared unfit for further use by the designated veterinarian (Art 14), killing will be done according to EU guidelines. Animals will only be re-used if they, at the end of the study, are suitable for other experiments covered by an existing CCD license or studies that are below threshold; if not, we will sacrifice them.

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

For studies 1 and 4 and pilot study, killing by decapitation is required for brain collection. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible.

For studies 2 and 3, killing by perfusion is required for brain collection. Intraperitoneal overdose of pentobarbital for deep anaesthesia and non-recovery will be used.

For study 5, F1 animals will be sacrificed after completing the behavioural tests and/or breeding as they have fulfilled the scientific purpose and their cumulative discomfort is classified as moderate. F2 animals of study 5 will be killed by decapitation for brain collection. Immediately prior to decapitation, animals will be sedated by inhalation of CO_2 and decapitation will be executed as fast as possible.

Companion females will be sacrificed after completing study 3 as they have fulfilled the scientific purpose and cumulative discomfort is classified as moderate.

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 will be used in F0 rats and non-used animals of all studies. It will be used also in companion females and F1 rats of study 5. The animal will be transported to a gas chamber in which CO_2 flow will be allowed until pressumed death is confirmed.
☒ Yes > Describe the method of killing that will be used and provide justifications for this choice.
Decapitation will be used in animals of the pilot study and studies 1, 4 and 5 to collect brain tissue and analyse gene expression level and epigenetic markers. Decapitation is selected because fresh frozen tissue samples are preferred to analyse gene expression level by using the quantitative polymerase chain reaction (RT-qPCR) technique. Prior to decapitation, the rat will be taken to a different room and will be exposed to CO_2 asphyxiation produced from dry ice until sedation. Immediately after, the animal will be decapitated by using a guillotine. Perfusion will be used in animals of studies 2 and 3 to collect brain tissue and analyse the c-Fos expression level. Perfusion is preferred to maximise good quality of brain slices to perform immunohistochemistry staining for c-Fos quantification. Prior to perfusion, the rat will be anaesthetised with an overdose of pentobarbital. Immediately after, a transcardiac perfusion with saline followed by 4% paraformaldehyde will be performed to clear blood and preserve the brain tissue.
If animals are killed for non-scientific reasons, justify why it is not feasible to rehome the animals.

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NIET-TECHNISCHE PROJECTSAMENVATTING

Naam van het project	Transgenerationele gevoeligheid voor depressie door stress in het vroege leven
NTS-identificatiecode	NTS-NL-285032 v.1
Nationale identificatiecode van de NTS *Veld wordt niet gepubliceerd.*	NTS202317174
Land	Nederland
Taal	nl
Indiening bij EU *Veld wordt niet gepubliceerd.*	ja
Duur van het project, uitgedrukt in maanden.	60
Trefwoorden	depressieve symptomen vroege stress serotonin transporter knockoutratten sociaal en affectief gedrag transgenerationeel
Doel(en) van het project	Fundamenteel onderzoek: Zenuwstelsel Omzettinggericht en toegepast onderzoek: Zenuwziekten en psychische aandoeningen van de mens

DOELSTELLINGEN EN VERWACHTE VOORDELEN VAN HET PROJECT

Beschrijf de doelstellingen van het project (bijvoorbeeld het aanpakken van bepaalde wetenschappelijke onduidelijkheden, of wetenschappelijke of klinische behoeften).	Depressie beïnvloedt het dagelijks leven door cognitieve stoornissen, angst en sociale disfunctie. De oorzaak is complex, waarbij genetische aanleg, omgevingsfactoren, en vroege stress een rol spelen. De omgeving voor, tijdens en na de zwangerschap is bepalend voor het welzijn van het kind op latere leeftijd. Als er een ongunstige omgeving is in de baarmoeder, of na de geboorte, heeft het kind een grotere kans om depressie te krijgen. Dit risico wordt verder verhoogd door kwetsbare genen. Het SERT-eiwit is zo'n kwetsbaar gen. Dit eiwit reguleert de werking van de zenuwoverdrachtsstof serotonine. Het gen dat de informatie bevat voor het aanmaken van SERT komt bij mensen voor in een korte en lange variant. De korte variant is gevoeliger voor het krijgen van een depressie, met name als dragers van deze korte variant meerdere ongunstige (stress) ervaringen ondergaan. Het is echter onbekend hoe de interactie tussen het hebben van een kort SERT-gen en tegenslag in het vroege leven een kwetsbaarheid voor depressie verhoogt. Om deze interactie te bestuderen zullen we ratten gebruiken die net als mensen met een kort SERT-gen, minder SERT-gen hebben. Als uit het onderzoek blijkt dat stress op jonge leeftijd bij deze ratten er inderdaad toe leidt dat de dieren symptomen van depressie gaan vertonen, spreken we van een proefdiermodel dat geschikt is voor onderzoek naar depressie. Recent werd aangetoond dat de kwetsbaarheid voor depressie hoger is als individuen blootgesteld worden aan vroege stressoren. Dit betreft de periode voor, tijdens, en na de zwangerschap. Om te onderzoeken of ratten ook depressie-achtige gedragingen ontwikkelen in combinatie met vroege stressoren zullen we in een verkennende studie (pilot) ratten blootstellen aan stress vlak voor, tijdens en net na de zwangerschap. Vervolgens zullen we cognitie, angst, depressie- en sociaal gedrag in de nakomelingen bestuderen. Als het proefdiermodel op deze manier gedefinieerd is, willen we het gaan gebruiken om onderliggende moleculaire mechanismen te ontrafelen. Vervolgens willen we onderzoeken of de gedragingen en onderliggende mechanismen doorgegeven worden aan de volgende generatie (transgenerationeel). Dit fundamentele inzicht is essentieel, niet alleen voor begrip van deze kwetsbaarheden, maar ook voor toekomstig onderzoek naar nieuwe behandelingen voor depressie, vooral bij (aanstaande) moeders.
Welke potentiële voordelen kan dit project opleveren? Leg uit hoe de wetenschap vooruit kan worden geholpen of mensen,	Als we een proefdiermodel kunnen ontwikkelen dat depressieve symptomen vertoont na aanpassing van het SERT-gen in combinatie met vroegtijdige tegenspoed (Early Life Stress) kunnen we onderzoek doen naar verschillende aspecten van depressie.

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).	

VOORSPELDE SCHADE

In welke procedures worden de dieren gewoonlijk gebruikt (bijvoorbeeld injecties, chirurgische procedures)? Vermeld het aantal en de duur van deze procedures.	Cumulatieve stress in het vroege leven (ELS). Wij trachten na te gaan welk type van cumulatieve ELS het sterkste effect heeft op het opwekken van depressie-achtige symptomen. Hiervoor zullen verschillende behandelingen van cumulatieve ELS toegepast worden op de dieren: - Net na de zwangerschap. Duur: de eerste twee weken van het leven van de nakomelingen na de geboorte (3 uur per dag). - Tijdens en net na de zwangerschap. Duur: de laatste week van de dracht van het vrouwtje (2 uur per dag) en de eerste twee weken van het leven van de nakomelingen (3 uur per dag). - Voor, tijdens en net na de zwangerschap. Duur: drie weken voor de zwangerschap van de vrouwtjes (onvoorspelbare duur elke dag), laatste week van de zwangerschap van de vrouwtjes (2 uur elke dag) en eerste twee weken van het leven van de nakomelingen (3 uur elke dag). Als de pilot studie laat zien nakomelingen van moeders met stress vlak voor, tijdens en net na de zwangerschap een depressief fenotype laat zien, willen we in vervolg bestuderen of hetzelfde effect in de nakomelingen ook te vinden is met minder stress in de moeder, om het ongerief zo laat mogelijk te houden. Stressors waar de ratten aan worden blootgesteld zijn: -Voor de zwangerschap: vrouwelijke ratten worden individueel gehuisvest en aan 1 a 2 stressoren per dag blootgesteld. Stressoren omvatten: 1 uur onder fel licht, overnacht overbezetting van ratten in een kooi, overnacht blootstelling aan vochtig bedding, 5 minuten zwemstress, kooirotatie voor 12 uur. -Tijdens de zwangerschap: In de laatste week van de zwangerschap worden vrouwelijke ratten drie keer per dag 45 minuten beperkt in hun bewegingsvrijheid (restraint stress). Ze bevinden zich in een plastic buis waarbij ze wel hun pootjes, maar niet hun lichaam kunnen bewegen. Dit gebeurd onder fel licht. -Na de zwangerschap: In de eerste twee weken worden de nakomelingen elke dag 3 uur per dag weg gehaald bij de moeder. Dit gebeurd op onvoorspelbare momenten gedurende de dag. In de 3 uur dat de pups weg gehaald worden, worden de moeders op onvoorspelbare momenten gestrest met een 5-minuut zwemstress test of met 20 minuten restraint stress.
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?	- Stress voor en/of tijdens de zwangerschap kan bij vrouwtjes het lichaamsgewicht verminderen, de bevruchting hinderen, voor miskramen zorgen en invloed hebben op het aantal nakomelingen dat geboren wordt of in leven blijft. - Stress net na de zwangerschap stress kan het sterftecijfer van de nakomelingen doen toenemen en/of kan het lichaamsgewicht van zowel de moeders als de nakomelingen doen afnemen. - Bij het testen van sociaal agressief gedrag zullen ratten gedurende 1 week sociaal geïsoleerd gehuisvest worden. Dit om het territorium af te bakenen voor de rat. Individueel huisvesten levert licht ongerief voor het dier. - Bij stress in de moeder wordt de moeder individueel gehuisvest. Individueel huisvesten levert licht ongerief voor het dier. - Als onderdeel van de stressor wordt 12 uur voedselrestrictie toegepast. Omdat dit gedurende de actieve fase is van de ratten, levert dit licht ongerief voor het dier.
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*
Ratten (Rattus norvegicus)	253	0	0	253	0

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.	De dieren worden na afloop van het experiment gedood voor onderzoek van de hersenen.

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.	In dit onderzoek willen we een proefdiermodel ontwikkelen voor depressie als gevolg van een verminderde SERT-gen in combinatie met Early Life Stress. Levende ratten zijn in staat een breed scala aan gedragingen te vertonen die functioneel vergelijkbaar zijn met die bij de mens. Tot op heden zijn er nog geen niet-dierlijke alternatieven ontwikkeld om gedragspatronen te bestuderen. Na afloop van de experimenten willen we moleculaire analyses uitvoeren in hersengebieden van het sociale hersennetwerk in de rat. Ook om die reden is vervanging niet mogelijk.
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.	In eerste instantie willen we een proefdiermodel ontwikkelen voor depressie als gevolg van een verminderd SERT-gen in combinatie met Early Life Stress. Voor experimenten die daarna volgen hebben zorgvuldig de benodigde groepsgrootte voor elk experiment berekend.
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.	Om de nadelige gevolgen van cumulatieve stress op jonge leeftijd te minimaliseren, zullen wij voldoende voedsel en water verstrekken om het lichaamsgewicht zo normaal mogelijk te houden. Bovendien zullen wij als kooiverrijking zorgen voor voldoende beddingmateriaal, knaagstokjes en groepshuisvesting. Dit helpt bij het omgaan met stress.
Licht de keuze van de soorten en de bijbehorende levensstadia toe	Cognitieve, affectieve en sociale gedragingen bij de rat vertonen functionele gelijkenissen met de mens. Bovendien zijn ratten, net als de mens, ook zeer gevoelig voor blootstelling aan stress vroeg in de ontwikkeling. Daarom kunnen veranderingen in het gedrag van ratten na Early Life Stress gelijkenis vertonen met menselijke gedragsveranderingen. Ook lijkt het erop dat hoe het zenuwstelsel bij ratten werkt voor sociaal gedrag, vergelijkbaar is met hoe het bij mensen werkt. We zullen de dieren testen als pups, als jonge en als volwassen dieren.

VOOR EEN BEOORDELING ACHTERAF GESELECTEERD PROJECT

Project geselecteerd voor BA?	ja
Termijn voor BA	31-08-2029

Reden voor de beoordeling achteraf

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

AANVULLENDE VELDEN

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

Advies aan CCD

B

Datum 10 november 2023
Betreft Advies Secretariaat over Aanvraag projectvergunning Dierproeven AVD202317174

Instelling:	5.1 lid2h
Onderzoeker:	5.1 lid2e
Titel project:	Unravelling the underlying mechanisms of early life stress induce disorders in animals with expression of the serotonin transporter
Aanvraagnummer:	AVD202317174
Betreft:	Nieuwe aanvraag
Categorieën:	Fundamenteel onderzoek Translationeel of toegepast onderzoek

1 Doelstellingen & belang van het project

Doelstelling	Citaat: The ultimate goal of this research is to determine whether the interaction of cumulative ELS and the SERT genotype increases the vulnerability to developing a depressive-like phenotype and to what extent the parental depressive-like phenotype is transferred to the next generation. Neurobiological mechanisms -including epigenetic markers-, depressive-like behaviours -including affective-related, cognitive and social behaviour- and transgenerational effects will be elucidated in the rat model. To reach this ultimate goal, immediate goals are proposed to be attained by conducting six studies: Phase 1: Pilot study Goal 1: To determine whether the combined exposure to (maternal) pregestational ELS, (maternal) prenatal ELS and postnatal ELS, ie cumulative ELS, induces a depressive-like behavioural phenotype in the rat. Goal 2: To determine whether the SERT+/- genotype increases the risk for depressive-like behaviours after the exposure to cumulative ELS. Phase 2: Studies 1 to 5 Goal 3: To assess the neurobiological mechanisms involved in the depressive-like phenotype induced by cumulative ELS. Goal 4: To identify whether social functioning and its neurobiological mechanisms are altered in young and adult rats exposed to cumulative ELS.

	Goal 5: To determine the extent to which the depression-like phenotype induced by cumulative ELS in one generation is transferred to the next and evaluate the neurobiological mechanisms involved. De doelen van het project zijn realistisch en haalbaar.
Wetenschappelijk en maatschappelijk belang	Citaat: This project addresses the influence of an environmental factor interacting with a genetic factor in the development of stress-related disorders. It also provides mechanistic insights in the effects of cumulative ELS on the development of stress-related disorders, especially in relation to the serotonin transporter genotype. Good animal models are indispensable in this research, as we can invasively study the underlying mechanisms in the brain of animals exposed to ELS mediated by the maternal stress (pregestational and prenatal), something that is not possible in humans. If the outcome shows that more rats express a depressive-like phenotype as a result of cumulative ELS, we will be able to elucidate the underlying mechanisms contributing to stress-induced disorders. In addition, if SERT+/- rats exhibit higher vulnerability to developing a depressive-like phenotype following the cumulative ELS exposure, we will be able to demonstrate that the serotonin transporter genotype moderates the influence of stress on depression. This would greatly benefit the clinical management of stress-induced disorders as new targets for drug treatment may be revealed. Additionally, if altered social behaviours are observed in adult and especially also in young animals exposed to cumulative ELS, we will be able to show a different neurodevelopmental trajectory of social functioning induced by cumulative ELS presumably related to the late onset of depression. Lastly, by studying if the parental depressive-like phenotype induced by cumulative ELS is transferred to the next generation, we will provide evidence of epigenetic signatures of the depressive-like phenotype shared between parents and the offspring.

Het belang is voldoende uitgewerkt en onderbouwd.

2 Kwaliteit Synthesis of Evidence

Kwaliteit SoE	Voorwerk is voldoende beschreven.
	Er is aannemelijk gemaakt dat het model geschikt is voor het gestelde doel.
Diermodel	Citaat bijlage: Affective-related, cognitive and social behaviours in the rat resemble functional similarities of the same behaviours in humans. Similar to humans, rats are also highly sensitive to stress exposure very early in development. Changes in rat behaviour following cumulative ELS can resemble human behavioural changes after a long history of ELS. In addition, the brain circuit for the expression of affective-related, cognitive and social behaviours is highly conserved across mammals. Therefore, brain circuit functionality in the rat resembles brain circuit functionality in humans. Citaat projectvoorstel: [...] Also, rodent studies fail to show solid evidence for increased vulnerability to developing depressive-like behaviour after early-life stress (ELS) in rodents with reduced SERT (heterozygous; SERT+/-) expression [15]. Therefore, the potential association of the SERT gene and psychiatric condition remains inconclusive. Nevertheless, previous studies show that the influence of 5-HTTLPR can be heterogeneous and highlight possible involvement of other factors and regulatory mechanisms promoting the risk of psychiatric disorders [16,17] [...] From this view, the exposure to cumulative ELS can interact with the SERT genotype -of the offspring- to increase the risk of depression. To our knowledge, no one has studied whether the onset of depression in the adult offspring is mediated by the interaction of cumulative ELS exposure and the SERT genotype.

3 3V's

Vervanging	Er is in voldoende mate onderbouwd dat de doelstelling niet zonder dieren behaald kan worden en voldoende duidelijk is ingegaan op het "nee, tenzij..." beginsel.
Vermindering & Verfijning	Er is niet in voldoende mate onderbouwd dat het project met zo min mogelijk dieren en zo verfijnd mogelijk wordt uitgevoerd. De onderzoeker geeft niet aan hoe het minimaliseren van het ongerief wordt gewaarborgd bij de monitoring van de procedures, zoals het blootstellen aan meerdere ELS stressoren. Hier zijn vragen over gesteld.

4 DEC advies

DEC-advies	Citaat vraag 7: The explanation in the appendix regarding cumulative discomfort is not sufficiently clear. In this argumentation, you should demonstrate that the cumulative discomfort remains within the moderate level. Furthermore, it is important to emphasize that a thorough evaluation of discomfort scores will take place during the initial phase, in close consultation with the IACUC (Institutional Animal Care and Use Committee). In this section, well-informed decisions can also be made regarding the continuation or termination of the research, based on solid reasoning. Citaat antwoord 7: We added the text below to the appendix. We hope it is clearer how we estimated the cumulative discomfort levels to 'moderate'. We realize that the experiments might be on the high end of the moderate discomfort levels, but still expect them within those levels. For the pilot study, we expect the pregestational, prenatal and postnatal stressors to be in the range of moderate. Especially because in our hands, the pre- and postnatal stressors have never led to a higher discomfort than 'moderate'. The combination of all three stressors is new, and we will monitor the animals closely and will contact immediately the Institutional Animal Care and Use Committee when the discomfort will be higher than anticipated (or when in doubt). Experiments will end (in consultation with the IACUC) when they exceed "moderate" discomfort levels. The animal behavior tests that the animals will undergo, are similar to what we have performed before in our lab and are in the range of mild. Cumulative discomfort for this group will therefore be estimated as 'moderate'. For all other groups, the behavioral tests we will use are tests we have ample experience with. In our hands, they never exceeded 'moderate' discomfort levels. The early life stressor with the most robust effects will be used, which may mean the pregestational, prenatal and postnatal stressors might be picked as the preferred model. We expect the discomfort of the mother to have an impact on the offspring, however we still expect this to be in the 'moderate' range when combined with the behavioral experiments (which are mild to moderate). Citaat C1: [...] Achtergrond, doelstelling en uitkomstparameters zijn duidelijk beschreven. De samenhang tussen de experimenten in de context van de doelstelling is helder en navolgbaar beschreven. Echter, de strategie is niet duidelijk genoeg beschreven. De criteria voor het go/no go beslismoment na het pilot-experiment zijn niet duidelijk vastgesteld. Het is niet duidelijk welke randvoorwaarden er nodig zijn om

	met de vervolg experimenten verder te gaan. Er staat bijvoorbeeld niet of een significante verandering in één of meerdere van de 7 gedragstesten nodig zijn om verder te gaan. Of de responders en non-responders worden meegenomen is evenmin duidelijk, alsmede hoe er wordt omgegaan met de verschillen tussen mannelijke en vrouwelijk dieren. Tevens is niet duidelijk of de studie beëindigd wordt (no go) wanneer de SERT +/- ratten geen additioneel verschil laten zien. 5.1 lid2h is op grond van bovenstaande van mening dat dit project toetsbaar is, overeenkomstig voorbeeld 1A van de handreiking 'Invulling definitie project'. Citaat C8: Bij C1 is de opzet van het project in verkorte vorm weergegeven. Voor dit project is het huidige diermodel op wetenschappelijke en praktische gronden gekozen. De opzet en uitkomstparameters sluiten aan bij de gestelde doelen. Echter, de strategie is hierbij niet geheel helder en/of logisch. Het is niet duidelijk voor welke uitkomstparameters het noodzakelijk is om een significant en robuust verschil te laten zien tussen experimentele groepen in de pilotstudie om daarna een vervolgstudie te gaan doen. Er is bijvoorbeeld niet beschreven of een enkele specifieke uitkomstparameter (zoals social play) voldoende is of dat één uit elke categorie voldoende is om verder te gaan. Met deze opzet is er wel een gerede kans dat binnen de looptijd van het project progressie gemaakt zal worden in het ontwikkelen van een diermodel dat een of meer depressieve symptomen vertoont na cumulatieve Early Life Stress in samenhang met een aanpassing van het SERT-gen. Echter of, en in welke mate, de nieuw verkregen kennis in dit ratmodel zal leiden tot het ontdekken van nieuwe medicijnen is de 5.1 lid2h niet geheel duidelijk. Citaat C10: De aanvrager heeft aangegeven dat verzorging en huisvesting van de dieren conform de richtlijn zullen zijn. Echter, dieren die in studie 3 een 'resident-intruder' test ondergaan, zullen 21 dagen individueel worden gehuisvest. Dit is uitvoerig beschreven en voor deze experimenten noodzakelijk. Het individueel huisvesten van de dieren leidt tot matig ongerief in ratten en is naar mening van de 5.1 lid2h voldoende onderbouwd.

Citaat C11:
Het is niet duidelijk of er bij deze aanvraag in rubriek F van de bijlage een realistische inschatting is gegeven voor het cumulatieve ongerief als maximaal licht voor 22% van de dieren en maximaal matig voor 78% van de dieren. Voor de pilotstudie is als inschatting gegeven voor het cumulatieve ongerief zijnde maximaal matig voor 100% van de dieren.

Het is volgens de 5.1 lid2h niet uit te sluiten dat er in de uiteindelijke proeven combinaties van handelingen zullen worden gebruikt die leiden tot een stapeling van ongerief op een zodanige wijze dat het in de bijlage beschreven cumulatieve ongerief van matig wordt overschreden. Dit geldt voor zowel de pilotstudie als alle vervolgstudies. De beschrijving van de procedures geeft een onvolledig overzicht en omdat er niet beschreven is hoe het maximaal cumulatief matig ongerief wordt gewaarborgd, acht de 5.1 lid2h de inschattingen van het (cumulatieve) ongerief twijfelachtig en mogelijk te laag.

Citaat C15:
[...] De go/no go strategie en de opbouw van experimentele groepen zijn niet geheel helder. Echter, op basis van de beschrijving in de aanvraag is de 5.1 lid2h van mening dat het aantal te gebruiken dieren realistisch is ingeschat en dat er voldoende getracht is om met zo min mogelijk dieren tot een betrouwbaar resultaat te komen. Het totale aantal ratten voor een periode van 5 jaar is daarmee een realistische en haalbare schatting op projectniveau.

Citaat C16:
[...] Echter, de onderzoeker geeft bij de monitoring van de procedures, zoals het blootstellen aan cumulatieve ELS, niet aan hoe het minimaliseren van het ongerief wordt gewaarborgd.

De onderzoeker beschrijft dat de handelingen zo worden uitgevoerd dat overbodige stress bij de dieren wordt voorkomen en het leed beperkt blijft tot maximaal cumulatief licht ongerief voor 22% van de dieren en verwacht maximaal cumulatief matig ongerief voor 78% van de dieren. Echter gezien de aard en intensiteit van de individuele stressoren die cumulatief worden aangeboden aan de proefdieren is de 5.1 lid2h van mening dat er onvoldoende waarborgen zijn voor beperking van ongerief om deze procedure vrij te geven voor een project van deze omvang en het door de onderzoekers ingeschatte cumulatieve ongerief van maximaal matig voor de ruime meerderheid van de dieren.

Citaat C19:
[...] Ondanks dat niet van alle dieren de dodingmethode volledig is

	beschreven, gaat de 5.1 lid2h ervan uit dat alle dieren worden gedood door een voor de rat passende dodingsmethode conform bijlage IV van richtlijn 2010/63/EU. De 5.1 lid2h is van mening dat de aanvrager dit punt in voldoende mate wetenschappelijk heeft onderbouwd. Zie bijlage 1 punt K. Citaat C20: In studie 5 kunnen niet-gestreste F1-ratten worden aangeboden voor hergebruik voor educatieve doeleinden, omdat ongemak bij deze dieren als mild wordt geclassificeerd. Als ze echter door de aangewezen dierenarts ongeschikt worden verklaard voor verder gebruik, worden ze gedood volgens de EU-richtlijn (deze methode wordt echter niet beschreven). Dieren worden alleen voor hergebruik aangeboden als ze aan het einde van het onderzoek geschikt zijn voor andere experimenten, die onder een bestaande CCD-vergunning vallen of studies die onder de drempelwaarde liggen. Om hoeveel eventueel te hergebruiken dieren het gaat is niet beschreven in de bijlage noch in de NTS. Ethische afweging van de DEC: Citaat D1: In dit advies worden twee morele vragen beantwoord. De 5.1 lid2h koppelt de pilotstudie los van de rest van de studie: 1) Rechtvaardigen de doelstellingen van het project 'Transgenerational susceptibility to depression due to early life stress' het cumulatief maximaal licht (22%) tot matig (78%) ongerief dat de proefdieren wordt aangedaan in het onderhavige project? 2) Rechtvaardigen de doelstellingen van de pilotstudie van het project 'Transgenerational susceptibility to depression due to early life stress' het cumulatief maximaal matig (100%) ongerief dat de proefdieren wordt aangedaan in het onderhavige project? Zoals onder C1 uiteengezet acht de 5.1 lid2h de aanvraag toetsbaar op project niveau en een ethische afweging is daarom mogelijk. Citaat D2:

	Waarden die voor de proefdieren in het geding zijn: de integriteit en het welzijn van de dieren zullen worden aangetast door een genetische verandering (SERT +/- ratten), blootstelling aan herhaaldelijke (cumulatieve) stressoren (ELS) alsmede aan 'intruders', een zevental gedragstesten, anesthesie, herhaaldelijke s.c. injecties en bloedafnames, individuele huisvesting en leven met aan depressie gerelateerde verschijnselen. De dieren zullen hiervan ongerief en stress ondervinden. Aan het eind van de proeven worden (de meeste zo niet alle) de dieren gedood. Algemeen: waarden die voor onderzoekers bevorderd worden zijn het beschikken over een diermodel dat depressief-achtige symptomen vertoont veroorzaakt door cumulatieve Early Life Stress en verstoring in de serotonine huishouding door een genetische verandering in het heropname mechanisme van serotonine om de neurobiologische mechanismen die betrokken zijn bij een depressief-achtig fenotype te kunnen bestuderen in de rat; verder, in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie. Dit kan in de toekomst mogelijk resulteren in inzicht in de opbouw, ernst en karakteristieken van de door ELS opgebouwde depressie in samenhang met een verstoorde serotonine huishouding en eventueel aangrijpingspunten voor nieuwe behandelmethoden tegen deze vorm van depressie mogelijk maken. De 5.1 lid2h is van mening dat de belangen van de samenleving in het algemeen en de wetenschap in het bijzonder binnen de pilotstudie van het project 'Transgenerational susceptibility to depression due to early life stress' zwaarder wegen dan de belangen/waarden van de dieren. Indien de doelstellingen van de pilotstudie bereikt worden, kan dit leiden tot de ontwikkeling van een diermodel dat later gebruikt kan worden voor de bestudering van cumulatieve ELS-effecten op depressief-achtig gedrag bij ratten, in samenhang met een verstoring in de serotonine huishouding door een genetische verandering in het heropname mechanisme van serotonine. Met dit diermodel kan in verder onderzoek ook bestudeerd worden in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie. Vanuit dit perspectief is de 5.1 lid2h van mening dat het onderhavige onderzoek wetenschappelijk van substantieel belang is. Het is aannemelijk dat de doelstellingen van deze pilotstudie behaald kunnen worden. Bij deze aanvraag heeft de 5.1 lid2h onder andere gesproken over het

	nut en de noodzaak van het onderzoek, de strategie, go/no go criteria, de 3 V's, de intensiteit van de stressor behandelingen in relatie tot het mogelijk cumulatief ongerief, de navolgbaarheid van de lotgevallen van de individuele dieren en het aantal benodigde dieren. Met de antwoorden op de vragen van de 5.1 lid2h en de gereviseerde versie van de aanvraag hebben de aanvragers deze (en andere punten) voldoende geadresseerd om de pilotstudie te vergunnen, maar heeft de 5.1 lid2h een probleem met het op voorhand verlenen van toestemming voor de pilotstudie in combinatie met de vervolgstudie om redenen van onduidelijkheden en onzekerheden in het protocol zoals boven genoemd. Citaat D3: 1. De 5.1 lid2h beantwoordt de centraal morele vraag: rechtvaardigt de doelstelling van het project 'Transgenerational susceptibility to depression due to early life stress' dat is gericht op het verkrijgen van een nieuw diermodel om te kunnen onderzoeken welke neurobiologische mechanismen ten grondslag liggen aan de invloed van cumulatieve ELS op het serotoninesysteem dat een belangrijke rol speelt bij depressie het cumulatief maximaal licht (22%) tot matig (78%) ongerief voor de ratten in het voorliggende project ontkennend. 2. De 5.1 lid2h beantwoordt de centraal morele vraag: rechtvaardigt de doelstelling van de pilotstudie van het project 'Transgenerational susceptibility to depression due to early life stress' dat is gericht op het verkrijgen van een nieuw diermodel dat cumulatieve ELS geïnduceerde depressieve verschijnselen vertoont in samenhang met een verstoring in de serotonine huishouding door een genetische verandering in het heropname mechanisme van serotonine het cumulatief maximaal matig (100%) ongerief dat de proefdieren wordt aangedaan in de pilotstudie bevestigend. Hoewel de 5.1 lid2h de intrinsieke waarde van het dier onderschrijft en oog heeft voor het te ondergane ongerief van de proefdieren, weegt het potentiële substantiële belang van de pilotstudie in dit project naar haar mening zwaarder. De volgende overwegingen hebben bijgedragen tot deze conclusie: • Indien de doelstellingen van de pilotstudie bereikt worden, zal dit kunnen resulteren in een nieuw diermodel waarin door cumulatieve ELS geïnduceerde depressie-achtige gedragingen kunnen worden bestudeerd. Daarnaast wordt vastgesteld of het hebben van een serotonine transporter (SERT)+/- genotype het risico op depressief gedrag verhoogt na de blootstelling aan cumulatieve ELS; verder, kan daarna bestudeerd

	worden in hoeverre het depressief-achtige fenotype wordt doorgegeven aan de volgende generatie. • Het is voorstelbaar dat eventuele vervolgonderzoeken kunnen leiden tot wetenschappelijke kennis en inzichten op het gebied van moleculaire mechanismen, die ten grondslag liggen aan de invloed van cumulatieve ELS op het serotoninesysteem en daarmee op depressie-achtige gedragingen bij de rat wat vervolgens misschien kan leiden tot een beter begrip van hoe verschillende early life stressoren psychische veranderingen kunnen veroorzaken bij de mens. De 5.1 lid2h is van mening dat de gekozen strategie en experimentele aanpak van het project als geheel ten dele onlogisch overkomen en niet helder aansluiten bij de aangegeven doelstellingen. De 5.1 lid2h is van mening dat de voorgestelde experimentele opzet van enkel de pilotstudie en betreffende uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstelling en naar verwachting zullen leiden tot het behalen van de doelstelling van de pilotstudie ter vaststelling van de onderzoeksparameters voor de vervolgstudies. De onderzoekers beschikken over de benodigde kennis en technische expertise om het voorgestelde experimentele werk goed uit te voeren. Om de doelstellingen van de pilotstudie te bereiken is het nodig om proefdieren te gebruiken. De onderzoekers schrijven dat ze er alles aan doen om het lijden van de ratten te beperken, waardoor het uiteindelijke (cumulatieve) ongerief van elk individueel dier, in de pilotstudie, naar verwachting beperkt blijft tot maximaal matig (100%). Er zijn naar mening van de 5.1 lid2h echter onvoldoende aanwijzingen om aan te nemen dat bij de uitvoering van de studies het maximale gedaan wordt om het cumulatieve ongerief te beperken tot maximaal matig, waardoor ernstig ongerief niet kan worden uitgesloten en zelfs kan worden verwacht in de pilotstudie. Op grond van deze overwegingen beschouwt de 5.1 lid2h de voorgestelde dierproeven in uitsluitend de pilot van het projectvoorstel 'Transgenerational susceptibility to depression due to early life stress' als ethisch gerechtvaardigd en voorziet de 5.1 lid2h derhalve enkel de pilotstudie van het onderhavige projectvoorstel van een positief advies en dientengevolge ontbeert de aanvraag voor alle overige onderdelen een (positief) advies. De DEC heeft extern advies ingewonnen bij

- de aanvrager
In de vergadering van 17 augustus heeft er een open gesprek plaats gevonden tussen de aanvrager en de 5.1 lid2h . De aanvrager heeft de vragen schriftelijk beantwoord en de aanvraag aangepast.

Het DEC advies is Verlenen onder voorwaarden
Citaat E1:
De 5.1 lid2h adviseert uitsluitend voor de pilotstudie de vergunning te verlenen.

Citaat E2:
[...] Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten:
De uitkomsten van de pilotstudie moeten gerapporteerd en beoordeeld worden. Daarin moet duidelijk worden dat:
1) Het ongerief dat de dieren in de pilotstudie ervoeren cumulatief maximaal matig was,
2) Bij welke van de 7 gedragstesten er een significant en robuust effect te zien was door de specifieke (cumulatieve) ELS,
3) Dat SERT +/- ratten een hogere kwetsbaarheid voor depressief gedrag vertonen dan SERT +/+ ratten als gevolg van cumulatieve ELS,
4) De data verkregen van de responders en non-responders worden meegenomen in de uiteindelijke conclusies van het onderzoek; dit betekent dat van de vrouwtjes die gestrest zijn bepaald moet worden wat hun niveau van stress is; dit is dan een co-variant in de analyses van de nakomelingen; dit tegen de achtergrond van de eerdere studies van deze groep (zie de antwoorden hierboven). [...]

Het uitgebrachte advies is niet gebaseerd op consensus.
Citaat E2:
De meningen van de 5.1 lid2h leden zijn verdeeld wat betreft de motivatie van de interventies, de logica van de vele interventies en of deze geminimaliseerd zijn. Het ongerief voor individuele dieren is redelijk duidelijk beschreven, maar niet dat er alles aan gedaan wordt om het maximale ongerief niet voorbij cumulatief matig te laten komen en ook niet hoe onnodig ongerief wordt voorkomen. De DEC-leden twijfelen mede daarom aan de correctheid van het te verwachten maximale cumulatief matig ongerief dat de aanvrager aangeeft.

Het advies is gebaseerd op een meerderheidsstandpunt.

	[...] Twee DEC leden kwamen tot een negatief advies ten aanzien van de gehele aanvraag inclusief de pilotstudie. Deze leden zijn van mening dat voor dit project de belangen van de proefdieren, die in het geding zijn, zwaarder wegen dan de belangen van de overige belanghebbenden. De redenen hiervoor zijn: • Naar de mening van deze beide leden moet het blootstellen van de betreffende dieren aan drie zogenaamde early life stressors (ELS) zowel voor, tijdens als net na de dracht worden geclassificeerd als cumulatief ernstig ongerief, terwijl niet uitgesloten kan worden dat zulks voor een tweetal early life stressors ook geldt. • Het twijfelachtig is of de resultaten van de studie bij de rat te extrapoleren zijn naar de mens daar de rat 5-HTTLPR niet tot expressie brengt. • De potentiële opbrengst in de vorm van (nieuwe) farmaca ten behoeve van behandeling van depressie bij de mens onvoldoende wordt geduid. • De lotgevallen van de individuele dieren nog steeds niet helder beschreven zijn. Concluderend: Op basis van de herziene aanvraag met de pilot en de beantwoording van de vragen zijn deze leden van mening dat er in de huidige versie onvoldoende grond is om te rechtvaardigen dat de doelstellingen van het project alsmede de voorgestelde en beargumenteerde uitvoering daarvan door de aanvrager zwaarder wegen dan het nu op de grens balanceren van cumulatief matig en ernstig ongerief van de ratten. Het voorgestelde project biedt in de huidige vorm onvoldoende perspectief voor nieuwe inzichten in de pathofysiologie en farmacotherapeutische behandeling van depressie bij mensen als gevolg van early life events in samenhang met variatie in de serotonine huishouding door een genetische verandering in het heropname-mechanisme van serotonine. De volgende dilemma's zijn gesignaleerd door de DEC: Citaat E3: Mogelijke onduidelijkheden en knelpunten zijn in twee vergaderingen besproken en ook met de onderzoekers gecommuniceerd tijdens een DEC-vergadering (zie vragen bij onderdelen A8 en de punten genoemd bij D. Ethische afweging). Naar het oordeel van de meerderheid van de 5.1 lid2h zijn deze punten op bevredigende wijze opgehelderd voor het vergunnen van enkel de pilotstudie.

5 Kwaliteit DEC advies

Kwaliteit DEC-advies	Het DEC advies is helder en navolgbaar. In het DEC advies is op heldere wijze inzicht gegeven in de vragen die aan de aanvrager zijn gesteld. Bij de beantwoording van de beoordelingsvragen verstrekt u een heldere onderbouwing. De ethische afweging volgt op logische wijze uit de beantwoording van de C vragen. Bij C10 had het Secretariaat graag uw advies gelezen met betrekking tot het individueel huisvesten van vrouwelijke ratten in de pilot-studie.

6 Beschrijving dieren

Naam proef	**Diersoort**	**Stam**	**Aantal dieren**	**Herkomst**
3.4.3.1 Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.				
	Ratten (Rattus norvegicus)	Wistar, SERT+/+ and SERT+/-	3.382	Dieren die voor onderzoek gefokt zijn

Naam proef		
3.4.3.1 Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.	De humane eindpunten zijn voldoende beschreven.	
Ratten (Rattus norvegicus)	Ongerief: 78,0% Matig 22,0% Licht	Voor de pilotstudie is als inschatting gegeven voor het cumulatieve ongerief zijnde maximaal matig voor 100% van de dieren. Het cumulatieve ongerief voor de gehele bijlage wordt geschat op maximaal licht voor 22% van de dieren en maximaal matig voor 78% van de dieren. Omdat niet beschreven is hoe het maximaal cumulatief matig ongerief wordt gewaarborgd, zijn additionele vragen gesteld aan de aanvrager.

Naam proef	Worden de dieren gedood?	Doden volgens richtlijn?
3.4.3.1 Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.	Ja	volgens de richtlijn.

Huisvesting en verzorging anders dan Bijlage III Richtlijn

3.4.3.1 Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.

Vrouwelijke ratten zullen gedurende de pregestationele fase individueel gehuisvest worden gedurende 21 dagen. Ook bestaat de mogelijkheid dat de dieren aan een voederrestrictie worden onderworpen. Ratten die in studie 3 een 'resident-intruder' test ondergaan, zullen 21 dagen individueel worden gehuisvest.

Hergebruik

Er is geen sprake van hergebruik van dieren.

Locatie uitvoering experimenten	- Alle proeven vinden plaats in een instelling van een vergunninghouder. - Er zijn geen problemen bekend met de vergunninghouder.

7 Inzicht proces

Proces	Tijdens de behandeling van deze aanvraag heeft de DEC contact opgenomen met het Secretariaat en deze aanvraag als complex bestempeld. De behandeltermijn is hierdoor met 15 dagen verlengd. Er zijn vragen gesteld over de NTS: - De NTS bevat over de gehele tekst en titel vaktermen (zoals, maar niet beperkt tot, neurotransmitter, postnatale, hersencircuit), waardoor de NTS voor het algemene publiek lastig navolgbaar kan zijn. Kunt u uw NTS nalopen op dergelijke termen en deze termen aanpassen of uitleggen? - In de bijlage dierproeven geeft u aan een pilotexperiment te zullen uitvoeren om de proefopzet van het hoofdonderzoek te optimaliseren. Kunt u het uitvoeren van het pilotexperiment opnemen in uw NTS? - In de bijlage dierproeven geeft u aan dieren individueel te huisvesten bij het afnemen van een 'resident-intruder' test. Het individueel

	huisvesten van dieren kan tot ongerief leiden. Kunt u individuele huisvesting opnemen in uw NTS? - In de bijlage dierproeven geeft u aan ratten te onderwerpen aan voederrestrictie. Het onderwerpen van dieren aan voederrestrictie kan tot ongerief leiden. Kunt u voederrestrictie opnemen in uw NTS? - In de NTS spreekt u over procedures waarin dieren cumulatieve stress krijgen. Kunt u inzichtelijk maken in uw NTS waar de dieren stress van krijgen? Er is een vraag gesteld over het projectvoorstel: - Kunt u de beantwoording van 3.2.3 opnemen in uw projectvoorstel? Er zijn vragen gesteld over de bijlage dierproeven: - Onder C. in uw bijlage dierproeven geeft u aan niet af te wijken van de richtlijnen beschreven in Annex III van de EU richtlijn. Kunt u hier het individueel huisvesten van ratten evenals de voerrestrictie opnemen in de toelichting? - Onder F. in uw bijlage is het individueel huisvesten van vrouwelijke ratten tijdens de pilot studie niet opgenomen in de ongeriefsclassificatie. Kunt u dit opnemen onder F. en het cumulatieve ongerief (indien noodzakelijk) aanpassen?

8 Samenvatting
Het projectvoorstel bevat voldoende informatie over het belang van het onderzoek en de humane eindpunten om tot een oordeel te kunnen komen. Het DEC-advies kan als grondslag dienen voor het besluit.

De DEC adviseert enkel de pilotstudie van het onderhavige projectvoorstel te vergunnen. Zij geeft hiervoor de volgende onderbouwing:
- De strategie is niet duidelijk genoeg beschreven;
- Criteria go/no-go beslismomenten niet duidelijk vastgesteld;

- Er is geen heldere onderbouwing hoe het maximaal cumulatief matig ongerief wordt gewaarborgd waarbij ernstig ongerief niet kan worden uitgesloten;

De aanvrager beschrijft het uitvoeren van pilotstudies om de proefopzet van het hoofdonderzoek te optimaliseren. Echter, het is niet geheel duidelijk welke uitkomstparameters uitgelezen worden en hoe deze zich verhouden tot criteria die nodig zijn om met vervolgexperimenten verder te gaan. Naar de mening van de DEC moeten de uitkomsten van de pilotstudie worden gerapporteerd en worden beoordeeld. 5.2 lid1

Voor de pilot-studie is een geschat cumulatief ongerief van maximaal matig voor 100% van de dieren gegeven. Omdat er niet beschreven is hoe het maximaal cumulatief matig ongerief wordt gewaarborgd, is de DEC voornemens een voorwaarde te stellen om de uitkomsten van de pilotstudie te rapporteren en te beoordelen. 5.2 lid1

Het DEC advies is gebaseerd op een meerderheidsstandpunt. Twee leden van de DEC hebben ieder een eigen minderheidsstandpunt ingenomen. Volgens deze leden zijn de belangen van de proefdieren in dit project van groter belang dan die van andere betrokken partijen omdat het blootstellen van de dieren aan drie "early life stressors" als ernstig ongerief wordt gezien. Zij twijfelen ook aan de relevantie van de resultaten voor menselijke toepassingen, benoemen het gebrek aan duidelijkheid omtrent het lot van de individuele dieren, en vinden dat de potentiële opbrengst van nieuwe medicijnen voor depressie onvoldoende wordt benadrukt.

De DEC kaart onder E3 van haar advies een dilemma aan, waarin wordt geschetst dat de leden van mening zijn dat de huidige versie van het project onvoldoende rechtvaardiging biedt voor de gestelde doelstellingen en de voorgestelde uitvoering ervan omdat het ongerief van de ratten nu op de grens van matig en ernstig balanceert. Ook biedt het project onvoldoende perspectief voor nieuwe inzichten in de behandeling van depressie bij mensen als gevolg van early life events in samenhang met genetische veranderingen.

5.2 lid1

De aanvrager beschrijft experimenten waarbij de ratten individueel gehuisvest zullen worden. In de pilot-studie worden vrouwelijke dieren gedurende 21 dagen individueel gehuisvest. Omdat dit gepaard gaat met het onderwerpen van de dieren aan verschillende stressoren binnen dezelfde tijdspanne, is hier een vraag over gesteld. In studie 3 worden de dieren maximaal 21 dagen individueel gehuisvest. De reden voor individuele huisvesting is het afnemen van een 'resident-intruder' test. 5.2 lid1 [redacted]

9 Voorstel besluit incl. voorstel geldigheidsduur van de vergunning

5.2 lid1 [redacted]

Beoordeling achteraf

Op dit project is een beoordeling achteraf van toepassing. Deze beoordeling zal uiterlijk augustus 2029 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

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.

Centrale Commissie Dierproeven

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5.1 lid2h
5.1 lid2e
5.1 lid2h

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Aanvraagnummer
AVD5.1 lid2h 202317174
Bijlagen
3

Datum 7 december 2023

Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte 5.1 lid2e

Op 4 juli 2023 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "Unravelling the underlying mechanisms of early life stress induce disorders in animals with expression of the serotonin transporter" met aanvraagnummer AVD5.1 lid2h 202317174. Wij hebben uw aanvraag beoordeeld.

Beslissing
Wij keuren uw aanvraag gedeeltelijk goed. Alleen de pilot-studie (Phase 1) in deze projectaanvraag wordt vergund. Hieraan worden aanvullende voorwaarden gekoppeld. Uit artikel 10a, eerste lid van de Wet op de dierproeven (hierna: de wet) volgt daarom dat het is toegestaan om de in de vergunning genoemde dierproeven uit te voeren binnen de gestelde vergunningsperiode. Deze vergunning wordt afgegeven voor de periode van 7 december 2023 tot en met 31 augustus 2028.

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

Beoordeling achteraf
Op dit project is een beoordeling achteraf van toepassing. Deze beoordeling zal uiterlijk augustus 2029 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

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

Procedure

Advies dierexperimentencommissie
Wij hebben advies gevraagd bij de 5.1 lid2h
(hierna: DEC). Dit advies is ontvangen op 2 november 2023. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel 10a, derde lid van de wet.

Nadere vragen aanvrager
Op 10 november 2023 hebben wij u om aanvullingen gevraagd. U heeft tijdig antwoord gegeven. Het verzoek om aanvullingen had betrekking op de Niet-Technische Samenvatting en de bijlagen dierproeven. Uw reactie is betrokken bij de behandeling van uw aanvraag.

Overwegingen
Wij kunnen ons vinden in de inhoud van het advies van de DEC. In aanvulling op het DEC-advies stelt de CCD de voorwaarde beoordeling achteraf. De voorwaarde is in de vergunning beschreven en is hieronder toegelicht. Ten aanzien van de overige onderdelen, nemen wij het advies van de DEC over, inclusief de daaraan ten grondslag liggende motivering.

Alleen de pilot-studie (Phase 1) in deze projectaanvraag wordt vergund. Bij een positief resultaat kan de rest van het onderzoek middels een wijzigingsaanvraag worden ingediend. Uit de uitkomst van de pilot moet komen vast te staan dat:

1. Het ongerief dat de dieren in de pilotstudie ervoeren cumulatief maximaal matig was;
2. Bij welke van de 7 gedragstesten er een significant en robuust effect te zien was door de specifieke (cumulatieve) ELS;
3. SERT +/- ratten een hogere kwetsbaarheid voor depressief gedrag vertonen dan SERT +/+ ratten als gevolg van cumulatieve ELS;
4. De data verkregen van de responders en non-responders worden meegenomen in de uiteindelijke conclusies van het onderzoek. Dit betekent dat van de vrouwtjes die gestrest zijn bepaald moet worden wat hun niveau van stress is.

Indien aan bovengenoemde vier voorwaarden wordt voldaan, beschouwt de CCD uitkomsten van de pilot studie als positief.

Op de volgende punten in de aanvraag zitten onduidelijkheden waardoor de aanvraag gedeeltelijk is afgewezen:

a. De strategie is onvoldoende helder beschreven, met onduidelijke criteria voor het go/no-go beslismoment na het pilot-experiment. Het is onduidelijk op basis van welke parameters de vervolgstudies worden gebaseerd. Om een vervolgstudie te rechtvaardigen dient geïdentificeerd te worden welke uitkomstparameters een significant en robuust verschil laten zien tussen experimentele groepen in de pilotstudie. Dit ontbreekt in uw project. Het is niet duidelijk of een significante verandering in één of meerdere van de 7 gedragstesten nodig is om verder te gaan. Of de responders en non-responders worden meegenomen is evenmin duidelijk, alsmede hoe er wordt omgegaan met de verschillen tussen mannelijke en vrouwelijk dieren. Door het ontbreken van een heldere strategie kan de CCD niet beoordelen of het dier in deze proef het minste ongerief zal ondergaan. De Wod vereist dat dit toetsbaar is. Als de CCD de toets niet kan uitvoeren, kan de CCD geen vergunning afgeven.

b. Door het ontbreken van een heldere strategie, go/no-go momenten, en het niet kunnen maken van een realistische inschatting voor het cumulatief ongerief, is het voor de CCD niet mogelijk om te beoordelen of de proeven zo verfijnd mogelijk worden uitgevoerd, en of het maximale gedaan wordt om het cumulatieve ongerief te beperken tot maximaal matig. De Wod vereist dat dit toetsbaar is. Als de CCD de toets niet kan uitvoeren, kan de CCD geen vergunning afgeven.

c. Door bovengenoemde punten is het voor de CCD niet mogelijk de schade en de baten van uw project tegen elkaar af te wegen. Deze afweging is echter wél verplicht op grond van artikel 10a2, tweede lid, onder d van de wet. Omdat de CCD deze wettelijk verplichte beoordeling niet kan maken, is de CCD gehouden uw aanvraag gedeeltelijk af te wijzen.

Beoordeling achteraf
Na afloop van het project moet er een beoordeling plaatsvinden zoals bedoeld in artikel 10a1, eerste lid, onder d van de wet. Er is geen heldere onderbouwing hoe het maximaal cumulatief matig ongerief wordt gewaarborgd. Omdat de CCD niet kan uitsluiten dat ernstig ongerief op zal treden, wordt een beoordeling achteraf opgenomen in de vergunning. Deze beoordeling zal uiterlijk augustus 2029 plaatsvinden. Meer informatie over de eisen die gesteld worden bij de beoordeling achteraf vindt u in de bijlage 'Weergave wet- en regelgeving'.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 93118, 2509 AC Den Haag.

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

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. Nadat u een bezwaarschrift heeft ingediend kunt u een voorlopige voorziening vragen bij de voorzieningenrechter van de rechtbank in de vestigingsplaats van de vergunninghouder. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisende situatie.

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

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl, stuur een e-mail naar info@zbo-ccd.nl of neem telefonisch contact met ons op: 0800 789 0789.

Centrale Commissie Dierproeven
namens deze:

5.1 lid2h

Algemeen Secretaris

Datum:
7december 2023
Aanvraagnummer:
AVD5.1 lid2h 202317174

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: 5.1 lid2h
Adres:
Postcode en plaats:
Deelnemersnummer:

deze projectvergunning voor het tijdvak 7 december 2023 tot en met 31 augustus 2028, voor het project "Unravelling the underlying mechanisms of early life stress induce disorders in animals with expression of the serotonin transporter" met aanvraagnummer AVD5.1 lid2h202317174, na advies van 5.1 lid2h De functie van de verantwoordelijk onderzoeker is Associate Professor. Het besluit is gebaseerd op de volgende (aangepaste) stukken:

1 een aanvraagformulier projectvergunning dierproeven, zoals ontvangen op 4 juli 2023
2 de bij het aanvraagformulier behorende bijlagen:
   a Projectvoorstel, zoals ontvangen op 14 november 2023;
   b Bijlagen dierproeven
      - 3.4.3.1 Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter., zoals ontvangen op 14 november 2023;
   c Niet-technische Samenvatting van het project, zoals ontvangen op 14 november 2023;
   d Advies van dierexperimentencommissie, zoals ontvangen op 2 november 2023
   e De aanvullingen op uw aanvraag, zoals ontvangen op 14 november 2023.

Naam proef	**Diersoort/ Stam**	**Aantal dieren**	**Ongerief**
3.4.3.1 Identification of behavioural, neurobiological, and transgenerational effects of cumulative early life stress in rats with reduced expression of the serotonin transporter.			
	Ratten (Rattus norvegicus) / Wistar, SERT+/+ and SERT+/-	253	100,0% Matig

Voorwaarden

Beoordeling achteraf
Op dit project is een beoordeling achteraf van toepassing. Deze beoordeling zal uiterlijk augustus 2029 plaatsvinden. Er zal dan conform artikel 10a2, derde lid van de wet, beoordeeld worden of de doelstellingen van het project werden bereikt.

Aanvraagnummer: AVD5.1 lid2h 202317174

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 lid2h202317174

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.

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

Artikel 10a2, tweede lid, onder d, van de wet
De projectbeoordeling omvat in het bijzonder een analyse van de schade en de baten die het project oplevert, waarbij wordt nagegaan of de schade in de vorm van pijn, lijden, angst of blijvende schade bij de dieren wordt gerechtvaardigd door het te verwachte resultaat met inachtneming van de ethische overwegingen, en op termijn voordelen kan opleveren voor mens, dier of milieu.