Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	50100
1.2	Provide the name of the licenced establishment.	TNO
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.4 Type of animal procedure: Long term effects of exposure to highly toxic chemicals and treatment

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

This study will be an extended version of appendix 3.4.4.3, in which the therapeutic efficacy at longer term will be evaluated. In case of exposure to highly toxic chemicals, the outcome on the long term may be detrimental for quality of life, for example due to brain injury resulting from oxygen deprivation or extensive epileptic insults. The main debilitating effects following such an exposure are therefore originating from brain injury, resulting in behavioural deficits and cognition deficiencies. Generally, lifesaving treatments are aimed at preventing such severe effects, thereby minimizing the long term effects. In case of high challenge doses, the severe effects may not always be completely mitigated, thus possibly resulting in long term functional adverse effects. In addition, effects may be induced by the medical intervention itself or in combination with the toxicant.
Certain groups of animals will be evaluated for specific parameters to identify factors affecting long term quality of life. These include:

1. Physiological parameters:
   - respiratory
   - cardiovascular
   - brain function and structure
2. Behavioral parameters
   - learning and memory,
   - anxiety
   - motor function
3. Histology and biochemistry:
   - at the end of the study, target tissues will be collected to be analysed biochemically or histologically.

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

Animals will be prepared for a chemical challenge and corresponding treatment as established in research covered in appendices 3.4.4.1 and 3.4.4.3. An appropriate in-life period representing a certain time period after chemical exposure and treatment will be designated, and can vary from 2-4 weeks to up to a year, if necessary. Depending on the number of parameters to be measured per animal, a time schedule will be designed, balancing the burden for the animals and the expected outcomes. Animals will be subject to tests maximally 3 times a day, with resting days in between if necessary.
The following behavioral and physiological tests following an initial test are envisaged:

1. **Physiological parameters**:

- **Respiratory**: Whole body plethysmography, max 24 hour monitoring per measurement. Measurement is performed as described in appendix 3.4.4.1 in a whole body plethysmograph.
- **Cardiovascular and EEG.** EEG and cardiovascular parameters will be tested in 4-24 hour periods, using telemetry. During this acquisition period, the animal cages will be placed on telemetric plates,. In certain cases, this can be combined with whole body plethysmography up to 24 hours.
- **Brain function and structure**
  MRI assessments (Magnetic Resonance Imaging) will be performed to analyze brain function and/ or structure. This technique will be performed at another establishment. To that end, animals will be transported by a dedicated courier. After reception at the establishment, animals will be housed in animal rooms, and allowed to acclimatize for a certain period before being subjected to repetitive MRI (minimal interval of 3 days between MRI, but in case of longer observation periods this interval will increase up to 3 months). Multiparametric MRI measurements will be conducted on a MRI system, under inhalation anesthesia, including mechanical ventilation, and appropriate monitoring of heart rate, oxygen and CO_2. All MRI protocols are up and running at the receiving establishment, and have been successfully tested and applied in small animal models of brain disease, including stroke, epilepsy and developmental disorders. The animals will be held at the MRI establishment until the end of the study.

2. Behavioural parameters

- Learning and memory,
  Morris Water Maze:
  Testing for learning and memory capability in small animals (mice, rats, guinea pigs) will be done in a Morris Water Maze. This test consists of a training phase to find a hidden platform in a water bath and a retrieval phase. Each training consists of 4 trials of maximally 90 seconds of swimming, the whole procedure generally consists of 5-7 training daily sessions. After recovery, 1 retrieval trial of 90-120 seconds of swimming can be recorded without an island in the maze. After retrieval, a retraining phase can be established, in which the island is placed on the other side of the swimming pool, and a training phase is started again.
  Shuttle box
  The shuttle box is an active avoidance test in which animals have to learn to avoid an aversive stimulus in response to a warning signal. An aversive stimulus is a brief mild footshock (rats) or air puff (guinea pigs), which is initiated after a visual signal (rats) or white noise (guinea pigs)
- Anxiety; motor function
  Open field
  General behavior (anxiety, motor behavior/ activity) can be assessed in an open field, in which spontaneous behavior of an animal can be registered during 5-15 minutes.
  Both water maze and open field are analyzed offline using video analysis software.
  Novelty Challenge
  The novelty challenge is a paradigm in which anxiety related behaviour can be analysed in rats. Animals are placed into a novel environment, consisting of a plastic cage, and their behaviour is recorded and scored for anxiety related behaviour over two consequent ~10 minute periods. Behaviour is scored afterwards using video analysis.

3. Histology and biochemistry:

At the end of the study, target tissues will be collected to be analysed biochemically or histologically. This can be certain specific receptor expression, markers for tissue injury, in particular in the brain

(inflammatory markers, neuronal injury)

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

In general, for behavioural assessments, and due to variable response to challenge and treatment, the variation within groups can be quite large, and thus larger animal groups are generally required. In case of long term studies, in particular in instrumented animals, the number of animals in which technical issues could be encountered will increase over time and could lead to a number of animals not fit enough to reach the end of the study. To ensure large enough groups over time, such loss has to be accommodated for in the experimental design. Additionally, measuring an increased number of parameters on the one hand increases the value of each animal, but on the other hand, multiple parameters require higher numbers of animals to correct for multiple comparisons. For each specific study design, a power analysis will be performed to calculate the minimum number of animals required to obtain a statistical valid result. Animal numbers will be corrected for anticipated loss over time, and for multiple comparisons depending on the number of parameters to be measured. In some cases, tissues or organs are required at predefined time points. In such cases, larger groups of animals will be assigned to treatments, and parts of the group will be sacrificed over time.
A typical study design for an extended study is provided below. At each time point, a set of parameters will be assessed as described above.

Group	Agent dose	Conventional treatment	Treatment dose	Number of animals/time point						
				24 h	1 week	1 month	3 months	6 months	9 months	
Time matched controls	0	0	0	12	12	12	12	12	12	*72*
Positive control	sub lethal*	conventional dose	None	12	12	12	12	12	12	*72*
Treatment Low	sub lethal	conventional dose	Low **	12	12	12	12	12	12	*72*
Treatment Mid	sub lethal	conventional dose	Mid **	12	12	12	12	12	12	*72*
Treatment High	sub lethal	conventional dose	High **	12	12	12	12	12	12	*72*
Total				*60*	*60*	*60*	*60*	*60*	*60*	***360***

***) To be determined from Appendix (3.4.4.1) experiment or literature**
****) To be determined from Appendix (3.4.4.3), literature or earlier experience**

B. The animals

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

For foundation: see appendix 3.4.4.1. Based on the research question the small animal model as described in appendix 1 will be used (mice, rats, guinea pigs). The use of large animals (minipigs) in this phase is not anticipated at this moment as this would require development and/ or implementation of behavioral assays.
Depending on the target population for registration, juvenile, adult or aged animals will be used. The choice of gender will be based on literature, but also in line with the sex to be used in the efficacy phase. Initial phases will most likely be performed in one gender only, in line with results obtained in studies performed in the framework of Appendix 3.4.4.1., taking into account whether the response to pharmaceutical is gender specific or not. However, to minimize possible variations, initial studies will preferably be performed in one gender at a time, and in follow-up stages critical challenges and doses might be transferred to the opposite sex.
Estimated numbers in 5-year project period:

Annex IV	Mice	Rats	Guinea pigs	Minpigs
Total estimated 5-year	40	400	200	0
Number of studies	1	2-3	1-2	0
1 Terminal	0%	0%	0%	0%
2 Mild	40%	0%	0%	0%
3 Moderate	60%	80%	80%	0%
4 Severe	10%	20%	10%	0%

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

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

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

☒ No

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

D. Replacement, reduction, refinement

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

An alternative approach in the sense of *in vitro* or *ex vivo* experiments is not possible since the parameters to be measured (motor activity, learning and memory capability) are complex and are related to the complexity of the brain and general physiology. Additionally, this appendix describes the final stages of intervention efficacy testing. This will always be preceded by earlier research stages, of which the primary stages have consisted of mechanistic target research, including *in vitro* assessments (such as target affinity, expected pharmacokinetics, etc.).
With regard to numbers and refining, optimization of multiple parameter measurements while keeping the animal burden as low as possible is always included in the experimental design.

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

Pre-challenge surgery will be performed under anaesthesia. Humane end point criteria will be determined to minimize the duration of animal suffering while maintaining scientific value.
The administration of substances and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that of themselves will result in no more than moderate discomfort. 'A Good Practice Guide to the Administration of Substances and Removal of Blood, Including Routes and Volumes'(Diehl *et al*) will be used as guidance.
The number and duration of behavioral and physiological measurements will be carefully chosen, with appropriate periods in between to ensure that animals have appropriate recuperation time, and interference of test results from different measurements is minimized.

Repetition and duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

☐ No

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

Before any intervention, animals will be housed according to the guidelines, or also whenever possible.
In cases in which animals are instrumented by surgery, animals will be housed individually to prevent damage to cannulas or electrodes.
In case of individual housing, social interaction is limitedly possible, due to the use of open cages which allow sniffing and reaching out to neighboring cages. Appropriate cage enrichment will be available for long term housing, such as wooden chewing blocks, paper rolls, play houses, shelters etc., always taking into account that the animals cannot hurt themselves.

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Not applicable

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

Surgical procedures will be carried out under species appropriate anesthesia, such as isoflurane (4-5% induction, 1-2% maintenance) anesthesia, depending on surgical procedure and animal species. Specific anesthesia and analgesia this will be specified in the individual study plan.
Appropriate peri-operative care will be provided with analgesics and/ or antibiotics up to 48 hours.
Animals will be monitored frequently immediately after surgery until full recovery, based on voluntary movement and consciousness, then lowered to twice daily, or once at later stage (after ~1 week). See humane endpoints for observation criteria.

I. Other aspects compromising the welfare of the animals

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

Animals might not recover well from pre-challenge surgery. Additionally, animals might suffer from long

term progressive toxicity, which could be intermittent seizure activity in case of nerve agent poisoning (with day to week intervals), or shortness of breath in case of inhalatory agents.
For MRI measurements, transportation (by road) of animals to another animal facility is required.
Individual housing up to 1 year. See also F.

Explain why these effects may emerge.

Although surgeries are always performed by well-trained technicians or scientists, certain procedures are not without risk. The long term effects of a toxic challenge depend on the initial challenge dose, and the efficacy of a countermeasure. In this phase of the research, the treatments to be pursued have been tested at shorter term and are expected to be accompanied with low discomfort, or improvement of the animals over time.
Animals will have to be transported by road to another facility in case of MRI assessment.
Individual housing is necessary when the animals just have undergone surgery, or for longer time periods when they are equipped with complex head stages.

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

In case of observing the effects of a medical intervention after a chemical challenge, the severity is already minimized as much as possible within the scientific boundaries. However, progressive deterioration of an animal is not aimed for, and will be circumvented by application of humane endpoints as described below. In general chemical challenges will be as low as possible, without compromising the scientific aim.
Transportation time kept as short as possible (within 2 hours, and will be performed by a dedicated courier). Acclimatization time after arrival will be a week for long term in life studies, and could be shorter for MRI assessment under terminal anaesthesia.
In case of long term physiological assessment, telemetric devices will be implanted, not requiring complex head stages. This will allow shorter individual housing (until recovery from surgery).

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

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

In addition to endpoints and observations in relation to pre-challenge surgery, as described in appendix 1, 2, 3, animals in this appendix may suffer from long term effects from a toxicant. Additionally, for certain long term studies, animals could show signs of old age, which might compromise the well-being of the animal negatively.
After a chemical challenge, the expected body weight loss can increase up to 20%, lasting for 1-3 days. If the animals fail to recover to their pre-challenge weight within 5 days, or show progressive weight loss during 3-4 days, euthanasia will be considered. This could be the case if signs of toxicity are progressive and not culminated or mitigated within the first 24-48 hours. An example for such a condition would be continuous seizures, or progressive labored breathing.
Furthermore a drop in heart rate under ~35% of baseline level is an indication for early termination of the experiment, which is expected only during acute challenge/ treatment phases. Furthermore, if certain toxicants induce severe toxic effects, such as ongoing seizures for >24 h, this will lead to application of the humane end point, based on the observation criteria a weight loss of the animals will be indicative for taking.

In case progressive deterioration occurs, as described above, and in line with general criteria in 3.4.4.1, animals wil be taken from the study in consultation with the Animal Welfare Body.

Indicate the likely incidence.

Additional risks are e.g. clogging of cannulas, or technical difficulties during measurements, failure of transmitters (electrodes). In case a medical countermeasure was not sufficiently effective or recovery is slower than anticipated based on the short term outcomes from stage 3.4.4.3, animals might be subjected to an early termination from the experiment. As the outcomes of stage 3.4.4.3 are expected to be predictive for the likely absence of longer term suffering, this is expected to be unlikely in this stage.

Per experiment, those risks will be specified to determine the number of reserve animals to be included in the study plan.

K. Classification of severity of procedures

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

	Annex IV	Species				Discomfort			
	Procedure	Mice	Rats	Guinea pigs	Minpigs	1 Non recovery	2 Mild	3 Moderate	4 Severe
Preparation	Cannulation		x	x				x	
	Butterfly access						x		
	EEG electrode placement		x	x				x	
	ECG electrode placement		x	x				x	
	Implantation of osmotic minipump	x	x	x			x		
Challenge	subcutaneous injection	x	x	x			x		
	intravenous injection	x	x	x			x		
	intramuscular injection		x	x			x		
	dermal application (percutaneous)		x	x			x		
	Inhalation		x	x			x		
	subcutaneous injection	x	x	x			x		
Treatment	intravenous injection	x	x	x			x		
	intramuscular injection		x	x			x		
	Intraosseus		x	x				x	
	Intraperitioneal	x	x	x			x		
Monitoring	Blood sampling		x	x			x		
	Telemetry		x	x			x		
	Subclinical		x	x			x		
	Sublethal		x	x				x	x[o]
	MRI					x	x [+]		
	Euthanasia		x	x		x			
	Individual housing >1 week		x					x	
Behavior	Morris Water Maze	x	x	x			x	x[*]	
	Open Field	x	x	x			x		
	Shuttle box		x	x			x		
	Novelty Challenge		x				x		

*: for guinea pig and possibly mice up to moderate

o: Moderate to severe, depending among others on the duration of the study. Long term individual housing is considered severe discomfort. Animals will be challenged initially with a highly toxic dose, but

are expected to recover to normal feeding and motor behaviour within a few days. Discomfort in the form of pain will be minimal, and be mitigated with analgesics in case of pre-challenge surgery. As described before, countermeasures that have shown at least partial effectiveness will be pursued at this stage, preventing severe signs, even in a sublethal challenge model (so, moderate to severe discomfort). Smaller groups of control animals could be part of the study, that might suffer from more severe signs of toxicity, with regard to short term severity and slower recovery, depending on the challenge dose. Repeated behavioural testing is considered mild discomfort.
+: MRI measurements will be performed under inhalation anaesthesia (mild), in certain cases non recovery (1).

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

Tissues will be collected for post mortem histochemical analysis.

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