European Commission

Caring for animals
aiming for better science

DIRECTIVE 2010/63/EU ON PROTECTION OF ANIMALS USED FOR SCIENTIFIC PURPOSES

© Novo Nordisk

RETROSPECTIVE ASSESSMENT RESULTS

Environment

Luxembourg: Publications Office of the European Union, 2024

Print ISBN 978-92-68-13031-5 doi: 10.2779/94804 KH-02-24-265-EN-C

PDF ISBN 978-92-68-13046-9 doi: 10.2779/896767 KH-02-24-265-EN-N

Update to the Non-technical Project Summaries under Article 43(2) of the Directive 2010/63/EU on the protection of animals used for scientific purposes

RETROSPECTIVE ASSESSMENT RESULTS

National Competent Authorities for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes

A working document on Submission of an Update to the Non-technical Project Summaries with Retrospective Assessment Results

Brussels, 10-11 October 2023

The National Contact Points of the Member States responsible for the implementation of Directive 2010/63/EU on the protection of animals used for scientific purposes ('the Directive') and the Commission agreed to discuss the practical implementation of the requirement under Articles 38 and 43 of the Directive with a view to finding a common approach throughout the EU.

Regulation (EU) 2019/1010 of the European Parliament and of the Council of 5 June 2019, amending Article 43 of the Directive, and the related Commission Implementing Decision 2020/569/EU set out legally binding common formats and data content for non-technical project summaries (NTS) and the results of retrospective assessment of projects (RAR). The previously endorsed Working document on NTS was updated and endorsed by the National Competent Authorities for the implementation of Directive 2010/63/EU at their meeting of 25-26 November 2021. Using the previously adopted approach, this further guidance on the update of the NTS with the RAR complements the earlier guidance and was endorsed by the National Competent Authorities at their meeting of 10-11 October 2023.

Table of Contents

Introduction

One of the key aims of the Directive is to enhance transparency and to ensure that the public is objectively informed about the use of animals for scientific purposes. This is clearly expressed in both Recital 41 and Article 43 of the Directive. The main tool that is used for this purpose is the publication of non-technical project summaries (NTS) and the retrospective assessment results (RAR) of authorised projects.

In 2013, the European Commission issued a working document with the aim of harmonising the approach to the completion of NTS within the European Union. In order to further improve transparency and to establish an open access EU database on the use of animals for scientific purposes, some amendments were made by means of Regulation (EU) 2019/1010 on the alignment of reporting obligations and Commission Implementing Decision 2020/569/EU (replacing Commission Implementing Decision 2012/707/EU).

The aim of the legislative changes is the creation of a central, open access, searchable database for NTS and related RAR (where required). Commission Implementing Decision 2020/569/EU (hereafter 'the Decision') sets out a common format for submitting this information to the Commission. To achieve the objective of enhancing transparency, and realise the maximum benefits of publishing NTS, harmonisation in the reporting of NTS and the RAR across Member States is essential. The updated guidance *Working document on Non-technical Project Summaries* was endorsed by National Competent Authorities in November 2021.

This document aims to guide on the updating of the NTS with the RAR. Thus, the objective is to provide guidance for competent authorities tasked with the retrospective assessment using the new template defined in Annex I Part B of the Decision. This is reproduced in this document (Appendix I). Illustrative examples of NTS updated with the RAR are provided in Appendix II. The examples are grouped in the three categories of projects referred in Article 39.

1. Using non-human primates
2. Containing severe procedures
3. Otherwise selected for retrospective assessment during project evaluation

Legal background

Non-technical project summaries and results of retrospective assessment

Article 39
Retrospective assessment

1. Member States shall ensure that when determined in accordance with Article 38(2)(f), the retrospective assessment shall be carried out by the competent authority which shall, on the basis of the necessary documentation submitted by the user, evaluate the following:

- *(a) whether the objectives of the project were achieved;*
- *(b) the harm inflicted on animals, including the numbers and species of animals used, and the severity of the procedures; and*
- *(c) any elements that may contribute to the further implementation of the requirement of replacement, reduction and refinement.*

2. All projects using non-human primates and projects involving procedures classified as 'severe', including those referred to in Article 15(2), shall undergo a retrospective assessment.

3. Without prejudice to paragraph 2 and by way of derogation from Article 38(2)(f), Member States may exempt projects involving only procedures classified as 'mild' or 'non-recovery' from the requirement for a retrospective assessment.

Article 43
Non-technical project summaries

1. Subject to safeguarding intellectual property and confidential information, the non-technical project summary shall provide the following:

(a) information on the objectives of the project, including the predicted harm and benefits and the number and types of animals to be used;

(b) a demonstration of compliance with the requirement of replacement, reduction and refinement.

The non-technical project summary shall be anonymous and shall not contain the names and addresses of the user and its personnel.

2. Member States may require the non-technical project summary to specify whether a project is to undergo a retrospective assessment and, if so, set out the deadline. In such a case, from 1 January 2021, Member States shall ensure that the non-technical project summary is updated within six months of the completion of the retrospective assessment with the results thereof[1].

3. Member States shall, until 31 December 2020, publish the non-technical project summaries of authorised projects and any updates thereto. From 1 January 2021, Member States shall submit for publication the non-technical project summaries, at the latest within six months of authorisation, and any updates thereto, by electronic transfer to the Commission.

4. The Commission shall, by means of implementing acts, establish a common format for submitting the information referred to in paragraphs 1 and 2 of this Article. Those implementing acts shall be adopted in accordance with the examination procedure referred to in Article 56(3). The Commission services shall establish and maintain a searchable, open access database on non-technical project summaries and any updates thereto.

Content and publication of the RAR to update the NTS

Article 39 of the Directive provides the key components that have to be included in the retrospective assessment. These have been broken down into sub-components for the RAR, which are detailed in Annex I Part B of the Decision. The template included in the Decision is attached here in Appendix I. Furthermore, the guidance *Working document on Project Evaluation and Retrospective Assessment* endorsed by National Competent Authorities in September 2013 explains the importance and the key elements of a good retrospective assessment.

However, additional guidance is considered helpful to aid competent authorities to draft meaningful, clear and succinct summaries of the retrospective assessment results, i.e. RAR, with a view to promote consistency within and between Member States and to enhance readability for the public.

Mandatory updating of the NTS with the RAR was optional. Only those Member States that, in their national legislation transposing the Directive, have stipulated that NTS shall specify whether a project is to undergo a retrospective assessment will be required to update the NTS with the RAR. In such cases, the NTS must be updated within six months of the completion of the retrospective assessment by the relevant competent authority. However, competent authorities in Member States where there is no legal obligation to update NTS with the RAR, may still choose to do so voluntarily. A list of the countries where updating NTS with RAR is mandatory is given in Appendix III (status of 2022).

The publication of the NTS and its RAR update can be performed using an electronic transfer to the European Commission that hosts an open-access, searchable EU database
https://webgate.ec.europa.eu/envdataportal/web/resources/alures/submission/nts/list

[1] Since July 2021 the EU ALURES NTS Database provides a link between the RAR and the respective NTS

NTS and the RAR can be prepared using any of the official Union languages. The EU database will hold the NTS and RAR in their original language. However, searches can be undertaken using any of the official Union languages. To facilitate this, certain terms from the templates will be available for searches in all official Union languages. In addition, an increasing number of intuitive keywords are expected to evolve over time on the basis of searches carried out. This will allow datamining of the entire database, irrespective of the language in which the NTS, or their updates, were submitted. It is for the user of the database then to translate the search results as necessary.

Benefits of publishing the RAR

A well-written RAR to update NTS can:

- Enhance openness and transparency around the use of animals in research.
- Present the outcome of the project against the objectives set, and, where applicable, the reasons where these were not achieved.
- Show actual versus predicted harms and explain the harms inflicted on the animals in a simple and easily understandable language.
- Show actual numbers of animals used versus estimates and provide potential reasons for variations.
- Provide adequate insight into the scientific and social (long-term) benefits of the project, while providing clarity if the benefits are to be expected as a result of cumulative projects. Explain possible reasons why expected benefits were not achieved/realised as originally predicted or as outlined in the NTS. This can also help to refine language about predicted benefits for future NTS so that the expected timescale is accurately reflected.
- Provide an analysis of reasons for (technical) problems that occurred and lessons to be learned from this. It is therefore important to share the outcomes regardless of the results.
- Support the sharing of good practices in relation to the principles of Replacement, Reduction and Refinement (the Three Rs) and identify new Three Rs applications for wider dissemination.
- Demonstrate that researchers and authorities have reflected on the authorised work and can apply improvements to quality and welfare in future studies.
- Be a critical evaluation similar to the discussion section of a scientific publication.
- Support evidence-based policy making by competent authorities.
- Facilitate improved design for similar studies, e.g., identify future refinement possibilities.
- Raise awareness of appropriate use and best practice.
- Raise awareness of animal uses and models that did not work as expected, thus contributing to the Three Rs
- Prevent others from repeating problems/mistakes.
- Assist competent authorities to review effectiveness of project evaluations/harm-benefit analysis.
- Improve the quality of the balanced scientific information available to the public and counter the spread of misinformation.

Guidance for the drafting of the RAR

The RAR is a summary of the retrospective assessment which itself may contain more information. RAR writers should be mindful that potential readers of the updated NTS may be people unfamiliar with scientific work. Only language and terminology which will be easily understood by the public should be used in the RAR. Any complex terms should be adequately explained.

Ensuring that the RAR update of the NTS is accurate and representative of the project

Public confidence in the benefits of publishing and updating NTS may be eroded if the information included within them is inaccurate, incomplete or uninformative. It is therefore important to ensure that the update is accurate and representative of the project. Goals/potential benefits and description of the project in the original NTS should be used, and text should build on that, or explain/discuss where it was not as anticipated.

Ensuring the safeguarding of intellectual property and confidential information

Article 43(1) requires that the NTSs shall be anonymous and that intellectual property and confidential information shall be safeguarded. Therefore, although not specifically stated in the Directive, it follows that the RAR update shall also be anonymous, shall not violate proprietary rights or expose confidential information.

Guidance on the content to be included in the template for the RAR

Information is required on whether the objectives of the project were achieved; the harms inflicted on animals, including the numbers and species of animals used, and the actual severity of the procedures; and any elements that may contribute to the further implementation of the requirement of replacement, reduction and refinement.

1) Achievement of objectives

Clear description of whether the objectives of the project were achieved and if there were other significant findings

- If achieved, how they have contributed to benefits of the project.
- If not achieved, or did not deliver the expected benefits, give an explanation and, if possible, include information on how the result can contribute to future projects.
- If applicable, include a description on any other significant findings that have emerged during the project development.
- Describe any amendments to the originally authorised project, if any.
- Include information on timing of further expected benefits.

Type of project

- Basic research
    - How did the project contribute to better understanding of the issue/increased knowledge/filling a knowledge gap? If possible, how were the results linked to a more tangible strategic goal, even though any wider benefits may be much further in the future and less predictable?
        - In which way did the project help to advance knowledge?
        - Did the results confirm the hypothesis to be scientifically sound and realistic?
    - How project results were disseminated, whether positive or negative, is particularly important for basic research to ensure the benefits are realised.
- Regulatory use/routine production
    - For testing or screening projects: indicate if the relevant regulatory guidelines or statutory requirements were met.
    - For service or production projects such as production of blood products/antibodies or new lines of genetically altered animals: state if demands for the service or product were met.
- Education/Training
    - When animals were used for education/training purposes, discuss e.g.:
        - The knowledge and skills required for surgeons and the importance to evaluate the success during the recovery phase.
        - If and how the knowledge and skills acquired by the trainees through this education/training are likely to be necessary for their future careers.

2) Harms

Information on the number, species and models used, including:

- Whether the numbers were different (higher or lower) from those originally estimated and an explanation why the numbers differed.

Severity of procedures in the project

- Allocation of actual severity of procedures, frequency and duration of procedures; explain what harms did the animals experience in a simple and easily understandable language. Where the actual severity levels were higher or lower than originally estimated, a detailed explanation should be provided, including potential reasons for the differences.
- When using animals for the purposes of education/training it is appropriate to limit the severity of procedures to "non–recovery" or "mild". However, it is recognised that well-justified exceptions could be accepted to this general principle. In those circumstances, it is important to describe why animals have been involved in procedures that resulted in higher severities.

Fate of animals kept alive at the end of the study

- Was there a difference between the fate of animals kept alive at the end of the study in comparison with the estimated? Explain reasons for the difference.

3) Any elements that may contribute to further implementation of the Three Rs:

General contribution to all of the Three Rs such as

- Have there been any developments in the Three Rs (including non-animal-based approaches) in the scientific field since the project was authorised? Can they be applied in this type of research? If not, why not?
- Could different approaches replace, reduce or refine further animal use?

Replacement

- How well was a tiered approach using e.g., non–animal alternatives, cadaver work and finally live animals implemented in education/training projects?
- What efforts were made to use non-animal derived materials and tools within the project?

Reduction

- Were the numbers of animals used appropriate for statistical analysis (too many/too few)?
- Were any new reduction strategies developed/implemented, especially within regulatory testing protocols and/or in routine production methods?
- If the project resulted in surplus animals (animals bred for but not used in procedures), could learnings from this project allow reducing the number of surplus animals in similar projects in the future? Please describe.

Refinement

- Were any new refinement techniques developed/implemented, especially within regulatory testing protocols and/or in routine production methods?
- Could animal monitoring regimes, housing and care conditions be improved?
- Were score sheets/welfare assessment protocols working well?
- Could humane end-points be refined?
- Could restraint methods be refined?
- Could anaesthesia, analgesia, and killing methods be refined?

4) Other

Dissemination of the Three Rs findings

- Has a description been given on how the findings for further implementation of the Three Rs will be disseminated?

Timing of the retrospective assessment/Duration of project:

- Project evaluators determine the most appropriate time for retrospective assessment on a case-by-case basis. If the timing of the retrospective assessment was not appropriate, the expected objectives might not have been achieved. In this case, explain to what extent the objectives set out in the application have been attained. It is worth noting that benefits in some cases may not be realised until sometime after the project has been completed e.g., basic research to increase knowledge. Language should reflect where harms are inflicted as a

result of the immediate project, but also reflect that benefits may only be realised as a result of cumulative projects.

Scale of project, complexity, novelty (e.g., new/pilot studies)

- If new models were introduced, or there were significant unknowns with regard to severity or effects on the animal, pilot studies may have been authorised. The competent authority may have determined that a retrospective assessment was undertaken on completion of such studies to ensure adequate consideration is given to the results, and further changes/measures introduced before more extensive studies were progressed. If this is the case, outcomes should be clearly described.
- Any other findings (referring to core scientific hypothesis, to methodological or animal welfare/animal medicine issues) considered relevant.

Appendix I – Template of RAR for NTS update

The template consists of all major information fields needed to update the NTS with the RAR and aims to research openness and support of public confidence.

Title (as per Non-technical Project Summary)			
Reason for Retrospective Assessment ([8])	**Using non-human primates**	**Contains severe procedures**	**Other reason**
Explain 'Other reason'			
Achievement of objectives			
Explain briefly whether, and to what extent, the objectives set out in the authorised project have been achieved. Provide reasons if objectives have not been attained. Have there been any other significant findings? What benefits have resulted from the work to date, and are further benefits expected? Have the results of this project been disseminated, including where hypotheses are not proven? If so, describe how. If not, indicate how and when results are expected to be publicised.			

Harms					
Species ([9])	**Total numbers of animals used**	**Numbers of animals per actual severity**			
		Non-recovery	**Mild**	**Moderate**	**Severe**

How do numbers of animals used and actual severities compare with those estimated? Where the actual numbers are higher than the estimated numbers, please provide an explanation. Where the actual numbers are lower, please provide an explanation unless that difference is a result of Reduction or Refinement?	
How does the fate of animals kept alive at the end of the study compare with the estimated fate? Please provide an explanation.	
Any elements that may contribute to further implementation of the Three Rs:	
1. Replacement	
With the knowledge obtained from this project, have any new approaches that could replace some or all of the use of animals in similar projects been identified/developed (including the development/validation of new *in vitro* or *in silico* techniques)?	
2. Reduction	
With the knowledge obtained from this project, could the experimental design be improved to enable any further reduction of the use of animals, and if so, how? Provide an explanation where numbers of animals used were lower than those originally estimated.	
3. Refinement	
Provide an explanation where the actual severities were lower than those originally estimated. With the new knowledge obtained from this project, are the animal models used still the most appropriate? Please specify per species/model, where appropriate. List any novel refinements introduced during the project to reduce harm to the animals or to improve their welfare. What are the potential opportunities for further refinement in the future, for example, emerging technologies, techniques, improved welfare assessment methods, earlier endpoints, housing/husbandry measures?	

4. Other	
How are the findings for further implementation of the Three Rs disseminated?	
Additional comments	

([8]) Multiple choices possible

([9]) Species in accordance with statistical reporting categories in Annex III to this Decision, with an additional option of 'non-specified mammal' to safeguard anonymity in exceptional cases

Appendix II – Illustrative examples of RAR

Illustrative examples of varying RAR with quality ratings are provided. NTS are also shown as reference.

1. Projects using non-human primates

Example 1 (good quality)

NTS

Title of the project	Neuromodulatory systems in the brain of primates
Duration of project (in months)	36
Keywords	Central Nervous System; Macaques; Neurological diseases; Neuroscience; Non-human primates
Purpose of project (multiple choices possible)	Basic research
Objectives and predicted benefits of the project	
Describe the objectives of the project (for example, addressing certain scientific unknowns, or scientific or clinical needs).	Neuromodulatory systems consist of small cell groups at the base of the brain, characterised by projection cellular structures, which orchestrate the proper functioning of the entire brain. This is illustrated by the fact that psychiatric and neurological diseases are associated with pathological changes in these cell groups. In this project we examine two of these cell groups: the *Area tegmentalis ventralis* (Transmitter dopamine) and the *Locus coeruleus* (Transmitter noradrenalin), whose organisation is not well known. By fine-anatomic examination of the two cell groups, we aim to 1) clarify their internal organisation and 2) their projections to the other parts of the brain. The latter is carried out by injection of so-called tracer substances, which can be absorbed by the nerve cells and transported to their projection sites and later detected under the microscope. We also aim to 3) investigate the influence of the two cell groups on other parts of the brain.
What are the potential benefits likely to derive from this project? Explain how science could be advanced, or humans, animals or environment may ultimately benefit from the project. Where applicable, differentiate between short-term benefits (within the duration of the project) and long-term benefits (which may accrue after the project is finished).	In order to be as close as possible to the understanding of the human brain, it is essential to carry out this study on non-human primates, as primates differ not only in their brain organisation but also in their cognitive abilities from those of other mammalian groups. The results will provide basic knowledge into the organisation and functioning of neuromodulatory systems (short-term benefits) and will provide an important basis for understanding thinking processes, emotional processing and perception, and their disease-related malfunctioning (long-term benefits).
Predicted harms	
In what procedures will the animals typically be used (for example, injections, surgical procedures)? Indicate the number and	Under general anaesthesia, a surgical operation is carried out which gives access to the brain through which cannulas or fine micro-electrodes are introduced. Small volumes of substances which activate nerve cells are injected through the cannulas, but the animals will be under anaesthesia. The procedure is similar to that of implanting a brain pacemaker on the human brain.

<table>
<tr><td>duration of these procedures.</td><td colspan="6">The procedure will be carried out once, and it will last about 5 hours under general anaesthesia.</td></tr>
<tr><td>What are the expected impacts/adverse effects on the animals, for example pain, weight loss, inactivity/reduced mobility, stress, abnormal behaviour, and the duration of those effects?</td><td colspan="6">The burden on animals is limited to the post-operational phase in which pain and wound treatment is carried out in the same way as in humans. The severity level is to be classified as moderate in accordance with Directive 2010/63/EU on the protection of animals used in scientific procedures.

We expect moderate post-operational pain for a couple of days (analgesics will be supplied). No behavioural abnormalities are expected, or inactivity or reduced mobility.</td></tr>
<tr><td rowspan="6">What species and numbers of animals are expected to be used? What are the expected severities and the numbers of animals in each severity category (per species)?</td><td>Species[4]</td><td>Estimated total numbers</td><td colspan="4">Estimated numbers per severity</td></tr>
<tr><td></td><td></td><td>Non-recovery</td><td>Mild</td><td>Moderate</td><td>Severe</td></tr>
<tr><td>Macaca fascicularis</td><td>5</td><td>0</td><td>0</td><td>5</td><td>0</td></tr>
<tr><td>Macaca mulatta</td><td>14</td><td>0</td><td>0</td><td>14</td><td>0</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>What will happen to the animals kept alive at the end of the procedure?[5, 6]</td><td colspan="2">Estimated number to be reused</td><td colspan="2">Estimated number to be returned to habitat/husbandry system</td><td colspan="2">Estimated number to be rehomed</td></tr>
<tr><td>Please provide reasons for the planned fate of the animals after the procedure.</td><td colspan="6">Two weeks after the introduction of micro-electrodes, the animals are killed for microscopic examination of the brain.</td></tr>
<tr><td colspan="7">Application of the Three Rs</td></tr>
<tr><td>1. Replacement
State which non-animal alternatives are available in this field and why they cannot be used for the purposes of the project.</td><td colspan="6">Because of the enormous complexity of the human brain organisation currently, non-animal methods (for example 3D brain-like structures or cerebral organoids) cannot yet replicate the details and complexity of the phenomenon under study. Rodents cannot be used in this study because their brain organisation is different from that of a primate (humans are primates).</td></tr>
<tr><td>2. Reduction
Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce the number of animals to be used, and principles used to design studies. Where applicable, describe practices that will be used throughout the project to minimise the number of animals used consistent with scientific objectives. Those practices may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.</td><td colspan="6">It is now possible for us to inject up to six different tracer substances during a single surgical operation on an animal, without this entailing a higher burden, apart from the slightly longer duration of the intervention. This significantly reduces the number of animals to be used. Thanks to this technique we hope we will be able to reduce the number of animals.</td></tr>
</table>

3. Refinement Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms to take up emerging refinement techniques during the lifetime of the project.	We use surgical methods, similar to deep brain stimulation in humans, and the same careful post-operational follow-up. The animals are inspected daily by the veterinarian and the technicians. If there are serious, untreatable and suffering complications in an animal, the animal shall be killed. Methodological improvements in surgical procedures and training help to minimise animal stress. In addition, we are collecting behavioural data of the test animals to enable targeted improvements. Furthermore, we will observe the applicability of less invasive examination techniques to reduce animal distress.
Explain the choice of species and the related life stages.	The macaque monkeys were specifically chosen because of their brain organisation but also their cognitive abilities. Humans and macaques share striking resemblances between their brain structures. Macaque monkeys are used as animal models in order to understand the mechanisms of the human brain. Adult animals are used (minimum 4 years) as most of these surgical methods are developed for adult macaque monkeys.

Project selected for Retrospective Assessment	Deadline	Contains severe procedures	Uses non-human primates	Other reason
			X	

Explanation of the other reason for retrospective assessment

RAR

Title (as per Non-technical Project Summary)	Neuromodulatory systems in the brain of primates		
Reason for Retrospective Assessment [8]	**Using non-human primates** **X**	**Contains severe procedures**	**Other reason**
Explain 'Other reason'	Not applicable		
Achievement of objectives			
Explain briefly whether, and to what extent, the objectives set out in the authorised project have been achieved. Provide reasons if objectives have not been attained. Have there been any other significant findings? What benefits have resulted from the work to date, and are further benefits expected? Have the results of this project been disseminated, including where hypotheses are not proven? If so,	The aim of this project was to examine pathological changes in small cell groups at the base of the brain as described in the non-technical summary. The aim of the project has been achieved within the predicted time. According to the researchers, the results of the project increase the basic knowledge of the internal organisation of the investigated cell groups and the projections and influence on the other parts of the brain. Further, the results obtained increase the basic knowledge of pathological changes and the malfunction of the observed cells and improve		

describe how. If not, indicate how and when results are expected to be publicised.	the understanding of the emergence of mental disorders, as well as psychiatric and neurological diseases. Some of the results have been already presented in two dedicated conferences. The data are now in preparation for two scientific publications in specialised journals. These journals apply the ARRIVE guidelines.

Species [9]	**Total numbers of animals used**	**Numbers of animals per actual severity**			
		Non-recovery	**Mild**	**Moderate**	**Severe**
Macaca fascicularis	5	0	0	5	0
Macaca mulatta	5	0	0	5	0

How do numbers of animals used and actual severities compare with those estimated? Where the actual numbers are higher than the estimated numbers, please provide an explanation. Where the actual numbers are lower, please provide an explanation unless that difference is a result of Reduction or Refinement?	Five Cynomolgus macaques (*Macaca fascicularis*) and 14 Rhesus monkeys (*Macaca mulatta*) were authorised. Thanks to a new tracing technique the research team was able to reduce the number of total animals utilised to 10 individuals, that is five Cynomolgus macaques and five Rhesus monkeys. The remaining individuals were just returned to the animal house. The moderate level of severity of the procedures did not differ from what was expected and was related to the surgical procedures under anaesthesia and post-operative pain. Analgesics were administered to reduce the pain of the animals. Animals that manipulated the wounds were separated for some days. No other harms than the one explained in the NTS occurred.
How does the fate of animals kept alive at the end of the study compare with the estimated fate? Please provide an explanation.	All animals utilised in the project (10) were killed at the end of the experiment, as previously indicated for microscopic examination of the brain.
Any elements that may contribute to further implementation of the Three Rs:	

1. Replacement	
With the knowledge obtained from this project, have any new approaches that could replace some or all of the use of animals in similar projects been identified/developed (including the development/validation of new *in vitro* or *in silico* techniques)?	No new alternative approaches were identified. Other mammals cannot be used due to significant differences in their brain organisation. Furthermore, in order to achieve the aim of the project, the researchers state that it is required to utilise an animal model which would mimic the integration between behaviour and the central nervous system, therefore an approach using a non-animal alternative would currently not be feasible.
2. Reduction	
With the knowledge obtained from this project, could the experimental design be improved to enable any further reduction of the use of animals, and if so, how? Provide an explanation where numbers of animals used were lower than those originally estimated.	As proposed in the project plan up to 6 different tracer substances per animal were injected within one surgery, without this entailing a higher burden, apart from the slightly longer duration of the intervention. This significantly reduced the number of animals. Therefore, thanks to this technique the research team was able to reduce the number of animals utilised from 19 to 10, while maintaining statistical significance.
3. Refinement	
Provide an explanation where the actual severities were lower than those originally estimated. With the new knowledge obtained from this project, are the animal models used still the most appropriate? Please specify per species/model, where appropriate. List any novel refinements introduced during the project to reduce harm to the animals or to improve their welfare. What are the potential opportunities for further refinement in the future, for example, emerging technologies, techniques, improved welfare assessment methods, earlier endpoints, housing/husbandry measures?	The level of severity was confirmed as being "moderate" as explained above. The *Macaca* spp monkeys continue to provide the most appropriate model for this kind of study. Positive reinforcement training was applied to increase the level of cooperation by the experimental subjects. The research team used state-of-the-art surgical methods and careful post-operational follow-up. The animals were inspected daily by the appointed veterinarian and during the week by the person responsible for animal welfare. No serious untreatable complications were observed. A dedicated technician has devised a series of easy-to-construct environmental enrichments, provided to the monkeys on a rotating schedule. The research team approved the applicability of magnetic resonance and ultrasound imaging as tools for less invasive examination procedures.
4. Other	
How are the findings for further implementation of the Three Rs disseminated?	The dissemination of the 3Rs Principle adopted in this project will be implemented by publishing the results

	of the project in journals which adopt the ARRIVE guidelines.
Additional comments	Summarised evaluation of the harms and benefits of the project. This was a basic research project, aimed at acquiring new knowledge on the functioning of the central nervous system. In particular, the aim of the project was to study the neuromodulatory systems of small cell groups of the brain, focusing on two groups of these cells. The results have provided basic insights into the organisation and functioning of neuromodulatory systems and will provide an important basis for understanding thinking processes, emotional processing and perception, and their disease-related disparity. The actual sources of harms were i) the restriction procedures imposed to the subjects to provide anaesthesia; and ii) post-surgical pain. In the first case a protocol of positive reinforcement training was adopted to avoid imposed physical restrain, and to assure collaboration of the experimental subjects for the injection; in the second case pain and wound treatments were provided to assure an adequate level of welfare of the animals involved. A system of remote recording with video cameras were used to check on animals manipulating the wound. In very few cases when this happened, the animals had to be restrained for a short time (through a sliding cage, to administer medicaments), and then kept separate for a couple of days. On the basis of this consideration, taking also into account that the project reached its aim in the predicted time, and the effort made by the research team to disseminate the results, the harm/benefit ratio can be considered satisfactory.

This is considered a good quality RAR because:

- **It is anonymous;**
- **Language is clear, concise and understandable to a layperson;**
- **The attainment of objectives was clearly described;**
- **It is explained that this use of animals is necessary and required in order to achieve the aims of the project;**
- **It is explained why the number of animals used was lower than expected;**
- **A summarised evaluation of the harms and benefits of the project is well described;**
- **It is explained how the application of the Three Rs was achieved:**
    - **Explained why Replacement was currently not possible and justification of the use of the animal species;**
    - **It is mentioned how the team managed the experimental procedures in order to reduce the number of animals previously planned to be used;**

- **Explained and listed some of the Refinements that were introduced.**

Example 2 (poor quality)

NTS

Title of the project	Heart development in primates		
Duration of project (in months)	60		
Keywords	Congenital heart disease; developmental biology; stem cells		
Purpose of project (multiple choices possible)	Basic research		
Objectives and predicted benefits of the project			
Describe the objectives of the project (for example, addressing certain scientific unknowns, or scientific or clinical needs).	The purpose of the study covered by the experiment is to study the embryonic heart development in the monkey embryo. We will use both normal embryos and monkey embryos that have genetic modifications that cause congenital heart defects. The foetus will be removed by caesarean section during the first third of pregnancy to study the development of the heart at the cellular level. By finding the specific problems in heart development that arise in animals with gene modifications, we get important information in trying to counteract these problems and, in the future, develop treatment strategies for congenital heart defects during the foetal period.		
What are the potential benefits likely to derive from this project? Explain how science could be advanced, or humans, animals or environment may ultimately benefit from the project. Where applicable, differentiate between short-term benefits (within the duration of the project) and long-term benefits (which may accrue after the project is finished).	We hope to better understand how the heart develops from its stem cells during the foetal period in primates - including humans. It is important to understand how heart malformations arise. Heart malformations are the most common form of malformations during the foetal period, and it is our hope that this application can lead to us becoming better at eventually predicting which human foetuses suffer from heart malformations and, in most cases, counteracting or treating these.		
Predicted harms			
In what procedures will the animals typically be used (for example, injections, surgical procedures)? Indicate the number and duration of these procedures.	The implantation of embryos is made using keyhole surgery or, if possible, with a thin cannula into the uterus with the help of ultrasound monitoring. All procedures, including caesarean section, are done under general anaesthesia and pain relief is administered to prevent discomfort after the surgery.		
What are the expected impacts/adverse effects on the animals, for example pain, weight loss, inactivity/reduced mobility, stress, abnormal behaviour, and the duration of those effects?	The expected negative effects are few. The caesarean section gives rise to a small wound, because the foetus is very small when it is removed. As a result, the harm will be of moderate severity.		
	Species[4]		**Estimated numbers per severity**

<table>
<tr><td rowspan="6">What species and numbers of animals are expected to be used? What are the expected severities and the numbers of animals in each severity category (per species)?</td><td></td><td>Estimated total numbers</td><td>Non-recovery</td><td>Mild</td><td>Moderate</td><td>Severe</td></tr>
<tr><td>Macaca fascicularis</td><td>12</td><td>0</td><td>0</td><td>12</td><td>0</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>What will happen to the animals kept alive at the end of the procedure?[5, 6]</td><td colspan="2">Estimated number to be reused</td><td colspan="2">Estimated number to be returned to habitat/husbandry system</td><td colspan="2">Estimated number to be rehomed</td></tr>
<tr><td>Please provide reasons for the planned fate of the animals after the procedure.</td><td colspan="6">After transabdominal embryo transfer, caesarean sections on the monkeys will be carried out up to 10 weeks after this. The embryos will be taken for analysis and the mothers can then be restored to full health.</td></tr>
<tr><td colspan="7">Application of the Three Rs</td></tr>
<tr><td>1. Replacement
State which non-animal alternatives are available in this field and why they cannot be used for the purposes of the project.</td><td colspan="6">Unfortunately, there are many differences between the development of the mouse and human heart. When it comes to heart malformations, which arise precisely during foetal development and are an important cause of illness in small children, it is therefore necessary to use laboratory animals, such as monkeys, that are biologically as close to humans as possible.</td></tr>
<tr><td>2. Reduction
Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce the number of animals to be used, and principles used to design studies. Where applicable, describe practices that will be used throughout the project to minimise the number of animals used consistent with scientific objectives. Those practices may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.</td><td colspan="6">A very large knowledge base has been built up with earlier studies in cell cultures and in mouse model systems. The embryos are planned based on this experience, and we can therefore, at a very early stage in the project, use embryos with exactly the same genetic defects that we expect will give rise to malformations. By taking out the foetuses at an early stage, we can implant four embryos in the same monkey and thereby keep the number of animals used down.</td></tr>
<tr><td>3. Refinement
Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms to take up emerging</td><td colspan="6">The development of the heart during the foetal stage differs between different animal species, and the development in rodents, rabbits and other common mammalian species is quite different from that in humans. It is therefore important to use an animal model where we know that the differentiated stem cells (progenitor cells) that are active during foetal development develop in the same way as in humans. Basic studies of these cells have been carried out on mice and in cell cultures since they were discovered about 10 years ago. However, getting ahead with clinical applications in humans, including treatment strategies for serious congenital heart defects, requires studies in monkeys, and crab macaques and rhesus monkeys are the primate species most commonly used in studies of the heart.</td></tr>
</table>

refinement techniques during the lifetime of the project.				
Explain the choice of species and the related life stages.	Humans and macaques share some similarities in foetal development. Given the need for a successful implantation and pregnancy, mature monkeys were necessary for the study.			
Project selected for Retrospective Assessment	Deadline	Contains severe procedures	Uses non-human primates X	Other reason
Explanation of the other reason for retrospective assessment				

RAR

Title (as per Non-technical Project Summary)	Heart development in primates		
Reason for Retrospective Assessment ([8])	**Using non-human primates** **X**	**Contains severe procedures**	**Other reason**
Explain 'Other reason'	Not applicable		
Achievement of objectives			
Explain briefly whether, and to what extent, the objectives set out in the authorised project have been achieved. Provide reasons if objectives have not been attained. Have there been any other significant findings? What benefits have resulted from the work to date, and are further benefits expected? Have the results of this project been disseminated, including where hypotheses are not proven? If so, describe how. If not, indicate how and when results are expected to be publicised.	The aim of the project was to map the development of the heart, including heart-specific stem cells, during the foetal period. This was to better understand and, in the long-term, treat congenital heart disorders with genetic background. Genetically modified embryos from collaborators in Kunming, China were used for *in vitro* fertilisation of foetal therapy. After a maximum of 10 weeks, the foetuses were taken for analysis by caesarean section. Although only one embryo could be isolated, the objective of the study was assessed as partially met. In the embryo, different types of progenitor cells (specialised stem cells) have been identified by *in vitro* techniques and sequencing has been carried out. Researchers stated that, together with data from partners, a detailed mapping of the development of the heart could be carried out.		
Harms			

Species ([9])	**Total numbers of animals used**	**Numbers of animals per actual severity**			
		Non-recovery	**Mild**	**Moderate**	**Severe**

Macaca fascicularis	12	8	0	4	0

How do numbers of animals used and actual severities compare with those estimated? Where the actual numbers are higher than the estimated numbers, please provide an explanation. Where the actual numbers are lower, please provide an explanation unless that difference is a result of Reduction or Refinement?	The analysis of the score sheets for four monkeys provided by the Proponents and Regional Ethics Committee confirmed the level of severity "moderate" for four of the experimental subjects. Eight monkeys were reported under "non-recovery" level of severity. In connection with the planned killing of monkeys in other experiments, these were transferred to the current permit for the removal of organs.
How does the fate of animals kept alive at the end of the study compare with the estimated fate? Please provide an explanation.	Four of the monkeys underwent transabdominal embryo transfer. In one of the animals this resulted in pregnancy and caesarean sections were carried out in week 6. The interventions were carried out under general anaesthesia and with post-operative pain relief. The suffering of the animals was assessed as being of moderate severity.
Any elements that may contribute to further implementation of the Three Rs:	
1. Replacement	
With the knowledge obtained from this project, have any new approaches that could replace some or all of the use of animals in similar projects been identified/developed (including the development/validation of new *in vitro* or *in silico* techniques)?	
2. Reduction	
With the knowledge obtained from this project, could the experimental design be improved to enable any further reduction of the use of animals, and if so, how? Provide an explanation where numbers of animals used were lower than those originally estimated.	

3. Refinement	
Provide an explanation where the actual severities were lower than those originally estimated. With the new knowledge obtained from this project, are the animal models used still the most appropriate? Please specify per species/model, where appropriate. List any novel refinements introduced during the project to reduce harm to the animals or to improve their welfare. What are the potential opportunities for further refinement in the future, for example, emerging technologies, techniques, improved welfare assessment methods, earlier endpoints, housing/husbandry measures?	The authorisation included three methods of embryo transfer. Of these, the method considered to be mild for the animal was used -transabdominal embryo transfer - where a catheter under ultrasonic monitoring is introduced through the abdominal wall into the uterus to deliver an embryo (at 4- to 8-cell stage). Methodology is not yet fully developed; however, the group of researchers also wishes to refine it as it is milder from the point of view of animal welfare than laparoscopy (key-hole surgery) which is commonly used.
4. Other	
How are the findings for further implementation of the Three Rs disseminated?	Established reporting standard. Data will be processed and presented in scientific articles.
Additional comments	Planned using the PREPARE guideline

Some information in this example is of good quality (e.g., the summarised evaluation of the harms and benefits of the project is well described).

However, the overall quality is considered poor because:

- **It is not anonymous;**
- **The attainment of objectives is vaguely described;**
- **It is explained why the number of animals used was lower than expected, but then the procedures are applied to only one individual, seriously compromising the significance of the results which is not discussed;**
- **Procedures performed are not clearly explained;**
- **The choice of the milder procedure is not clear at all;**
- **The application of the Three Rs Principle is not fully described;**
- **Further implementation of the Three Rs Principle is not described;**
- **The fate of the animals after the protocol is vaguely described.**

2. Severe procedure

Example 3 (good quality)

NTS

Title (as per Non-technical Project Summary)	Potency test of the *Clostridium chauvoei* fraction
Duration of project (in months)	60 months
Keywords	Batch potency testing; Vaccine; Efficacy; Clostridial Diseases in ruminants;
Purpose(s) of the project	Regulatory use and routine production – Quality control (including batch safety and potency testing)
Objectives and predicted benefits of the project	
Describe the objectives of the project (for example, addressing certain scientific unknowns, or scientific or clinical needs)	*Clostridium chauvoei* is considered as one of the most pathogenic bacteria amongst the *Clostridium* species. It is responsible for causing Blackleg, which is a global, severe, and mostly lethal bacterial infectious disease of ruminants, such as cattle and sheep. It spreads globally manifesting as a fulminant myonecrosis (the infection complications lead to muscle tissue death |necrosis|), oedemic lesions (swelling caused by the accumulation of fluid in a particular part of the body, for example, in legs), fever that is rapidly followed by lameness and generally leads to the death of the animals within a very short time. It causes significant losses in livestock with an enormous economic impact consequently. Due to its rapid progression and to the high mortality, which mostly is the only sign of the disease, antibiotics are generally not used to cure this infection. However, preventive measures by vaccination are efficient and farm health programs rely on vaccinating animals to control this disease, making the commercial production of effective vaccines an ever-present need. In the interests of animal welfare, the manufacturing of vaccines has to be subjected to a strict quality control and every batch of veterinary vaccines have to be tested in order to be shown to be in accordance with the standards of consistent quality, safety and efficacy before they can be released onto the market. The aim of our project is to comply with this regulatory requirement. We will receive batches of vaccines from a sponsor and will be testing batches of vaccines for them, in order to demonstrate that they are of high quality, stable and potent enough to induce a satisfactory immune reaction and protect cattle and sheep from the disease caused by *Clostridium chauvoei.* The potency test we are going to apply will use live animals (guinea pigs) and will be performed in line with the European Pharmacopoeia (Ph. Eur.) which is the legal basis for the quality requirements for medicinal products in Europe.
What are the potential benefits likely to derive from this project? Explain how science could be advanced, or humans, animals or environment may ultimately benefit from the project. Where applicable, differentiate between short-term	Conducting this regulatory quality test to batches of vaccines produced, will make it possible to only find on the market vaccines that have had their effectiveness checked, thus allowing to infer that they will induce a high standard of immunisation to animals (cattle and sheep) against the disease caused by *Clostridium chauvoei*.

<table>
<tr><td>benefits (within the duration of the project) and long-term benefits (which may accrue after the project is finished).</td><td></td></tr>
<tr><td colspan="2">Predicted harms</td></tr>
<tr><td>In what procedures will the animals typically be used (for example, injections, surgical procedures)?
Indicate the number and duration of these procedures</td><td>The test consists of using guinea pigs under a multi-step procedure consisting of a number of interventions.

Animals will be administered the first dose of the vaccine by subcutaneous route (injection under the skin). After 28 days the second dose of the vaccine will be administered by the same route.

14 days after the second vaccination, animals will be inoculated with a suspension of Clostridium chauvoei by intramuscular route (injection into the muscle tissue).</td></tr>
<tr><td>What are the expected impacts/adverse effects on the animals, for example, pain, weight loss, inactivity/reduced mobility, stress, abnormal behaviour, and the duration of those effects?</td><td>The animals can experience a slight discomfort and stress in relation to the injections. In addition, they might present some of the disease symptoms such as fever.

As animals may die after the infection challenge, severity experienced by animals is prospectively classified as severe.</td></tr>
<tr><td>What species and numbers of animals are expected to be used?
What are the expected severities and the numbers of animals in each severity category (per species)?</td><td>
<table>
<tr><th rowspan="2">Species</th><th rowspan="2">Estimated total numbers</th><th colspan="4">Estimated numbers per severity</th></tr>
<tr><th>Non-recovery</th><th>Mild</th><th>Moderate</th><th>Severe</th></tr>
<tr><td>Guinea pigs (Cavia porcellus)</td><td>4505</td><td>0</td><td>0</td><td>0</td><td>4505</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>
</td></tr>
<tr><td>What will happen to the animals kept alive at the end of the procedure?</td><td>
<table>
<tr><th>Estimated number to be used</th><th>Estimated number to be returned to habitat/husbandry system</th><th>Estimated number to be rehomed</th></tr>
</table>
</td></tr>
<tr><td>Please provide reasons for the planned fate of the animals after the procedure</td><td>All animals will be humanely killed at the end of the project.</td></tr>
<tr><td colspan="2">Application of the Three Rs</td></tr>
<tr><td>1. Replacement
State which non-animal alternatives are available in this field and why they cannot be used for the purposes of the project.</td><td>For the moment, there is no alternative method to assess the immune response in the animals. Given the large number of components of the immune system involved in the response in vivo, this test can currently not be reproduced in vitro. It is not yet possible to achieve the objectives of this project without the use of live animals because their use is required for regulatory purposes.</td></tr>
<tr><td>2. Reduction
Explain how the numbers of animals for this project were</td><td>The numbers of animals and the design of the procedures are according to the European Pharmacopoeia monograph Clostridium chauvoei vaccine for veterinary use (0361) we are going to follow.</td></tr>
</table>

determined. Describe steps that have been taken to reduce the number of animals to be used, and principles used to design studies. Where applicable, describe practices that will be used throughout the project to minimise the number of animals used consistent with scientific objectives. Those practices may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.	The group sizes of animals to use are 10 per batch of vaccine and 5 for the control group. In order to reduce the number of animals used, the batches of vaccines that we are going to test will be grouped as much as possible for being tested in parallel, having the same conditions and with the same group of control animals. We will use a ratio of 100 batches/1 control group.
3. Refinement Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms to take up emerging refinement techniques during the lifetime of the project.	The staff is well educated in laboratory animal science and the staff who will perform the procedures and monitor the animals is authorised, trained and competent in order to avoid unnecessary suffering to the animals. Additionally, regular literature reviews will be performed during the whole duration of the project to ensure that procedures being used are as refined as possible and remain in line with good practice recommendations. In order to safeguard welfare, animals will be monitored and scored frequently using limit points to ensure that no animal exceeds a pre-determined level of distress, and humane endpoints will be implemented immediately if any animal is found to be experiencing unexpected adverse effects. If signs appear that an animal is going to die from the infection it will be killed in advance.
Explain the choice of species and the related life stages.	The animal species we will use in the project, guinea pigs, is chosen in accordance with the standards required by the European Pharmacopoeia monograph *Clostridium chauvoei* vaccine for veterinary use (0361).

Project selected for Retrospective Assessment	Deadline	Contains severe procedures X	Uses non-human primates	Other reason
Explanation of the other reason for retrospective assessment				

RAR

Title (as per Non-technical Project Summary)	***Potency test of the Clostridium Chauvoei fraction***		
Reason for Retrospective Assessment ([8])	**Using non-human primates**	**Contains severe procedures** X	**Other reason**
Explain 'Other reason'	Not applicable		
Achievement of objectives			

Explain briefly whether, and to what extent, the objectives set out in the authorised project have been achieved. Provide reasons if objectives have not been attained. Have there been any other significant findings? What benefits have resulted from the work to date, and are further benefits expected? Have the results of this project been disseminated, including where hypotheses are not proven? If so, describe how. If not, indicate how and when results are expected to be publicised.	This was a test to verify if the produced vaccines are potent enough to protect cattle and sheep from disease caused by *Clostridium chauvoei*. The researchers found that the batches of vaccines they tested were potent enough to induce a high level of protective immunity. Thus, the objectives have been achieved, since the tests carried out on the vaccine batches have assured that they were of high quality. All product requirement standards were met, which has enabled their marketing. The project was carried out due to regulatory requirements that demand testing of a product before it is put on the market. The researchers got the batches from a sponsor and performed the test for them. The researchers communicate the results to the sponsor but are not publishing the specific results in other channels. However, they share general information at conferences on what they do within the 3Rs, for example the use of limit points to improve animal monitoring and how they managed batches in order to use less control animals.

Harms

Species [9]	**Total numbers of animals used**	**Numbers of animals per actual severity**			
		Non-recovery	**Mild**	**Moderate**	**Severe**
Guinea pigs (*Cavia porcellus*)	2010	0	1706	168	136

How do numbers of animals used and actual severities compare with those estimated? Where the actual numbers are higher than the estimated numbers, please provide an explanation. Where the actual numbers are lower, please provide an explanation unless that difference is a result of Reduction or Refinement?	The project was authorised to use 4505 guinea pigs but the number of animals used, 2010, was far lower than that because the sponsor produced fewer batches of vaccines than expected initially, which reduced the number of batches to be tested for stability. The actual severity of the procedures performed on animals is included in the mild, moderate and severe categories. 136 animals (of these 10 were control animals) died from the infection before the researcher could humanely anaesthetise them and were therefore classified in the severe category. 168 animals presented with fever, weight loss and stress behaviour and were therefore judging by the scoring sheets to be classified as having reached moderate severity. The remaining 1706 animals only experienced the injections with vaccine and inoculation with the *Clostridium* and were therefore classified as having reached mild severity.

How does the fate of animals kept alive at the end of the study compare with the estimated fate? Please provide an explanation.	All animals used were killed at the end of the project, according to the project plan.
Any elements that may contribute to further implementation of the Three Rs:	
1. Replacement	
With the knowledge obtained from this project, have any new approaches that could replace some or all of the use of animals in similar projects been identified/developed (including the development/validation of new *in vitro* or *in silico* techniques)?	The researcher reported that there were no results from the project that have or can lead to replacement. They also state that, for the moment there is no alternative method to assess the immune response in animals. Given the large number of components of the immune system involved in the response *in vivo*, the tests cannot yet be reproduced *in vitro*. The animal species was chosen in accordance with the standards required by the European Pharmacopoeia monograph *Clostridium chauvoei* vaccine for veterinary use (0361).
2. Reduction	
With the knowledge obtained from this project, could the experimental design be improved to enable any further reduction of the use of animals, and if so, how? Provide an explanation where numbers of animals used were lower than those originally estimated.	The researchers had 200 batches of vaccines to test. According to the European Pharmacopoeia, the group size of animals to be used per batch is 10 and for the control group is 5. In order to reduce the number of animals used, the batches of vaccines were grouped as much as possible for being tested in parallel, having the same conditions and using the same control group. In this respect, they used a ratio of 100 batches/1 control group, which made possible to reduce the number of control animals to only 10 (5 + 5). The researchers used both sexes in the experiment, therefore reducing the number of surplus animals resulting from this project.
3. Refinement	
Provide an explanation where the actual severities were lower than those originally estimated. With the new knowledge obtained from this project, are the animal models used still the most appropriate? Please specify per species/model, where appropriate. List any novel refinements introduced during the project to reduce harm to the animals or to improve their welfare. What are the potential opportunities for further refinement in the future, for example, emerging technologies, techniques, improved welfare assessment methods, earlier endpoints, housing/husbandry measures?	The tests were performed in accordance with the European Pharmacopoeia and therefore it was necessary to use guinea pigs and the experimental design planned as pointed out. Until the European Pharmacopoeia monograph that was followed is revised, this continues to be the most appropriate model to continue checking the quality of *Clostridium chauvoei* vaccine batches. The researchers state that the staff is continuously trained in improving the procedures and monitoring techniques in order to avoid additional suffering of the animals.

	Additionally, the staff attends scientific conferences regularly to keep up to date with new developments in the 3Rs area. Limit points are used to improve animal monitoring and are reviewed regularly in order to maximise the refinement. To avoid unnecessary suffering following the injection of *Clostridium*, animals that the researchers concluded were going to die from the infection were killed in advance. The cages where the animals are kept are enriched with tunnels and wood to gnaw on. The staff has trained the guinea pigs so handling and injections of the animals are less stressful than they would have been otherwise.
4. Other	
How are the findings for further implementation of the Three Rs disseminated?	The staff disseminates information on their 3Rs work at scientific conferences. Additionally, the sponsor is committed to help validating new *in vitro* tests in the future. The researchers state that European Pharmacopoeia is revising monographs for quality control of some other different *Clostridium* agents' vaccines in order to phase out animal testing. It is their hope that the concept will be able to be extended to all clostridial agents' vaccines monographs in a near future.
Additional comments	

This is considered a good quality RAR because:

- **Language is clear, concise and understandable to a layperson;**
- **It is anonymous;**
- **The attainment of objectives was clearly described;**
- **It is explained that this use of animals is necessary and required in order to meet regulatory requirements;**
- **The guideline that was followed to develop the project is referred to, in this case, a European Pharmacopoeia monograph;**
- **It is explained why the number of animals used was lower than expected and information on the reduction of surplus animals is specifically mentioned;**
- **It is explained how the Three Rs were applied:**
    - **Explained why Replacement was not possible and justification of the use of the animal species was added;**
    - **It is mentioned how the team managed the experimental groups in order to better apply Reduction;**

- **Explained and listed some of the refinements that were used / introduced.**

Example 4 (poor quality)

NTS

Title of the project	Evolutionary physiology of adaptation to multistress gradients of the moor frog
Duration of project (in months)	36 months
Keywords	Environmental stress; hormone measurement; acidification
Purpose of project (multiple choices possible)	Basic research - Ethology/Animal Behaviour/Animal Biology Preservation of species
Objectives and predicted benefits of the project	
Describe the objectives of the project (for example, addressing certain scientific unknowns, or scientific or clinical needs).	The aim of this project is to understand how natural moor frog populations are evolutionary adapted to physiological challenging ("stressful") environments. In particular, we want to understand how those populations are adapted to their local environment, especially, in relation to acidification, which is a stressor that may be of natural or human origin. It has been recently observed that different wild moor frog tadpole populations have adapted to different pH values, from acidic to neutral. Now, we will study the adaptation of tadpoles from different natural habitats along an acidic gradient, shaped as a combination of natural and human-induced acidification. Changes in pH may also influence the presence of predators and parasites in the habitats of moor frog tadpoles, which are potential additional stress factors and will be therefore considered in this project as well. We will analyse how they deal with (simultaneously) occurring stress factors by measuring stress hormone concentration and evaluating the occurrence of changes in morphology, growth and at genetic level. We plan to carry out the study under standardised laboratory conditions and in a more natural environment, in a mesocosm. A mesocosm is a reservoir of water that holds large parts of an ecosystem, can be conducted outdoors and allows the researcher to vary specific factors. For the hormone measurements, plasma has to be taken from the animals. Therefore, we want to prove whether changes in hormone levels can be measured in the surrounding water. This potential non-invasive method would reduce animal suffering, animal numbers and would make a significant contribution to the 3Rs in this research area.
What are the potential benefits likely to derive from this project? Explain how science could be advanced, or humans, animals or environment may ultimately benefit from the project. Where applicable, differentiate between short-term benefits (within the duration of the project) and long-term benefits (which may accrue after the project is finished).	The short-term benefit of this study is that it should contribute to scientists' understanding on how animals generally adapt to their environment and whether different populations within a species are genetically adapted to local conditions. In the long term, we expect the possible lessons to learn from this project can contribute to the development of strategies to protect endangered species.
Predicted harms	

In what procedures will the animals typically be used (for example, injections, surgical procedures)? Indicate the number and duration of these procedures.	Tadpoles will be divided into several experimental and two control groups and will be exposed to different combinations of the environmental stressors, in particular to acidic pH gradients (pH 4 and pH 7,5), parasites and to predators (dragon fly larvae). Plasma will be taken from the tadpoles once for hormone measurements. For the analysis of morphology and growth of living tadpoles, photographs will be taken.
What are the expected impacts/adverse effects on the animals, for example pain, weight loss, inactivity/reduced mobility, stress, abnormal behaviour, and the duration of those effects?	The animals will be exposed to the same stress factors they face in their natural environment, for the maximum duration of two weeks. Depending on the adaptability to the different stressors, the animals may experience stress of different degrees of severity. Especially, predation of dragon fly larvae in the mesocosm experiment will cause severe stress and increase mortality of tadpoles. Animals will be subjected only once to plasma sampling. The collection of plasma will last a few minutes and will be performed just prior to killing of the tadpoles. Thus, potential pain due to the sampling procedure will not last for an extended duration. The morphology and growth of tadpoles will be analysed from photographs, which will be taken multiple times on the same individual, but this procedure is not expected to cause any additional adverse effect on animals.

What species and numbers of animals are expected to be used? What are the expected severities and the numbers of animals in each severity category (per species)?

Species[4]	Estimated total numbers	Estimated numbers per severity			
		Non-recovery	Mild	Moderate	Severe
Other amphibians (Other Amphibia)	8000	0	0	4000	4000

What will happen to the animals kept alive at the end of the procedure?[5, 6]

Estimated number to be reused	Estimated number to be returned to habitat/husbandry system	Estimated number to be rehomed

Please provide reasons for the planned fate of the animals after the procedure.	All animals will be killed at the end of procedures, in order to harvest material for further morphological and genetic analyses.
Application of the Three Rs	
1. Replacement State which non-animal alternatives are available in this field and why they cannot be used for the purposes of the project.	This study aims to investigate how animals, in particular moor frogs, interact with and adapt to their environment (acidification, predators and parasites) and therefore relies on living organisms. There is yet no non-animal alternative known, that can reconstruct these complex interactions.
2. Reduction Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce the number of	A statistical calculation was performed to determine the number of animals that will allow scientifically robust data. Calculations are based on knowledge acquired over many years of experience of our team in the study of population variability and on relevant literature.

animals to be used, and principles used to design studies. Where applicable, describe practices that will be used throughout the project to minimise the number of animals used consistent with scientific objectives. Those practices may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.	
3. Refinement Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms to take up emerging refinement techniques during the lifetime of the project.	In order to ensure that no tadpoles exceed pre-determined level of stress or pain, they will be monitored several times a day. Animals experiencing unexpected suffering (e.g. injuries, diseases) or showing abnormal morphology or behaviour (e.g. swimming in circles, bent vertebral column, interrupted development and growth) will be humanely killed immediately. Plasma will be taken from the tadpoles using appropriate needle size. We think we will be successful in developing a new method for measuring stress hormone levels which we consider to becoming an important refinement alternative in the future. If it will be possible to measure the stress hormone in the surrounding water instead of taking plasma samples from the tadpoles, we will start using it immediately. This would reduce the planned number of animals for plasma sampling and reduce suffering of the animals. Furthermore, it would allow to measure the same individual multiple times, for example, during different development points. We will continuously review literature in order to identify new refinement possibilities that can be applied to the running project.
Explain the choice of species and the related life stages.	The aim of the present project is to gain better understanding of the adaptability of moor frog tadpoles to their environment. This can only be achieved using wild populations of this specific animal species and of this developmental stage.

Project selected for Retrospective Assessment	Deadline	Contains severe procedures X	Uses non-human primates	Other reason
Explanation of the other reason for retrospective assessment				

RAR

Title (as per Non-technical Project Summary)	***Evolutionary physiology of adaptation to multistress gradients in moor frog***		
Reason for Retrospective Assessment ([8])	**Using non-human primates**	**Contains severe procedures** **X**	**Other reason**
Explain 'Other reason'	Not applicable		

Achievement of objectives	
Explain briefly whether, and to what extent, the objectives set out in the authorised project have been achieved. Provide reasons if objectives have not been attained. Have there been any other significant findings? What benefits have resulted from the work to date, and are further benefits expected? Have the results of this project been disseminated, including where hypotheses are not proven? If so, describe how. If not, indicate how and when results are expected to be publicised.	The aim was to understand how natural moor frog tadpole populations are evolutionary adapted to physiologically challenging ("stressful") environments. So, the procedures of this project were planned to contribute to this animal species protection and preservation by increasing information on whether different populations are adapted to local conditions. Tadpoles from several wild populations, which had previously been proven to have adapted to acidic or pH neutral environments in several characteristics (including morphology and growth rate) were used. The study was mostly based on laboratory tests in which several characteristics, including the amount of a stress hormone in the whole organism and the amount of hormone transmitted into the water, were compared in studies with tadpoles from different populations under standardised conditions. A mesocosm experiment was conducted outdoors to explore adaptation in more ecologically relevant environments. Researchers reported that not all experiments have been conducted successfully for logistical reasons.

Harms

Species [9]	**Total numbers of animals used**	**Numbers of animals per actual severity**			
		Non-recovery	**Mild**	**Moderate**	**Severe**
Other amphibians (Other Amphibia)	6053	190	2333	3530	0

How do numbers of animals used and actual severities compare with those estimated? Where the actual numbers are higher than the estimated numbers, please provide an explanation. Where the actual numbers are lower, please provide an explanation unless that difference is a result of Reduction or Refinement?	6053 animals were used. For 3530 animals the degree of actual suffering was moderate. 190 tadpoles have been found dead.
How does the fate of animals kept alive at the end of the study compare with the estimated fate? Please provide an explanation.	No animal survived.

Any elements that may contribute to further implementation of the Three Rs:

1. Replacement

With the knowledge obtained from this project, have any new approaches that could replace some or all of the use of animals in similar projects been identified/developed (including the development/validation of new *in vitro* or *in silico* techniques)?	Plasma based measurements of the stress hormone can be replaced by the development of a water-based hormone measurement method.
2. Reduction	
With the knowledge obtained from this project, could the experimental design be improved to enable any further reduction of the use of animals, and if so, how? Provide an explanation where numbers of animals used were lower than those originally estimated.	Plasma based measurements of the stress hormone were partially replaced by a water-based hormone measurement.
3. Refinement	
Provide an explanation where the actual severities were lower than those originally estimated. With the new knowledge obtained from this project, are the animal models used still the most appropriate? Please specify per species/model, where appropriate. List any novel refinements introduced during the project to reduce harm to the animals or to improve their welfare. What are the potential opportunities for further refinement in the future, for example, emerging technologies, techniques, improved welfare assessment methods, earlier endpoints, housing/husbandry measures?	The moor frog remains an appropriate model for studying biological and ecological aspects of this animal species. No novel refinements were introduced.
4. Other	
How are the findings for further implementation of the Three Rs disseminated?	Water-based stress hormone measurements will be included in a methodological article that researchers are preparing.
Additional comments	

Reasons to consider this a poor example of RAR:

Some information in this example is of good quality (e.g., understandable language).

However, the overall quality is considered poor because:

- **It is not explained adequately whether, and to what extent, the objectives set out in the authorised project have been achieved:**
  - **It is stated that parts of the planned experiments have not been carried out, but no reasons for that are given.**
  - **It is described what has been done during the project. However, it remains unclear, if there are other findings that could lead to the attainment of all the**

other objectives that were prospectively planned, besides a newly developed refinement method (water-based hormone measurement technique).

- **The number of animals is lower than estimated in the application, but no adequate explanation for this is provided;**
- **It is stated that 190 animals were found dead. If death is related to the procedure, the actual reported severity shall be 'severe' unless an informed decision can be made that the severity can be assigned a lesser category. However, the assignment to the category "non-recovery" is incorrect;**
- **The newly developed non-invasive technique for hormone measurements should be considered and reported as a contribution to reduction and refinement, rather than as a replacement method.**

3. Projects otherwise selected for retrospective assessment during project evaluation

Example 5 (good quality)

NTS

Title of the project	Studying the mechanisms of brain development and their role in behavioural disorders
Duration of project (in months)	60 months
Keywords	Nervous system; postnatal development; neural pathways; autism
Purpose of project (multiple choices possible)	Basic research: nervous system Basic research: developmental biology
Objectives and predicted benefits of the project	
Describe the objectives of the project (for example, addressing certain scientific unknowns, or scientific or clinical needs).	The objective of the project is to further understand the role of specific genes that are contributing to the normal development of the brain in mammals including humans. It has been shown that these genes are still expressed in the brain after birth. Now we want to understand the role of these genes following birth. Furthermore, these genes are associated with neurodevelopmental disorders, such as ADHD, autism and learning disabilities, and certain types of cancer. Based on existing data we hypothesise that the genes of interest might regulate molecular pathways that are altered in for example autism spectrum disorders. In particular, it will be investigated how specific genetic changes affect the brain itself and behaviour.
What are the potential benefits likely to derive from this project? Explain how science could be advanced, or humans, animals or environment may ultimately benefit from the project. Where applicable, differentiate between short-term benefits (within the duration of the project) and long-term benefits (which may accrue after the project is finished).	The findings from this project could lead to a better understanding of the molecular mechanisms involved in the development and plasticity (the capacity of the nervous system to modify itself, functionally and structurally, in response to experience and injury) of neural circuits in the postnatal period. Indeed, the postnatal period is critical for the development of the brain and any alteration to these processes can be potentially a risk factor for neurodevelopmental diseases. Understanding the mechanisms is an essential requirement for diagnosis and the development of effective therapies.
Predicted harms	
In what procedures will the animals typically be used (for example, injections, surgical procedures)? Indicate the number and duration of these procedures.	1. In the first 3 days after birth, the genetically modified mice are to be injected with a hormone under the skin, in order to reduce the expression of the gene of interest. 2. The adult mice will undergo behavioural tests to evaluate their activity, motor strength, motor co-ordination, balance as well as social, repetitive and stereotypical behaviour to investigate loss of gene function. The mice will undergo two to three different tests. A test will last up to a few minutes. 3. Brains will be removed from some living animals, not previously used in the study, under anaesthesia and pain treatment. After assuring the sufficient depth of anaesthesia, the brain will be removed immediately. These animals will not recover consciousness.
What are the expected impacts/adverse effects on	1. Animals may experience mild pain at the site of hormone injection for a short time and these injections will be repeated four times.

<table>
<tr><td>the animals, for example pain, weight loss, inactivity/reduced mobility, stress, abnormal behaviour, and the duration of those effects?</td><td colspan="6">2. The majority of behavioural tests will induce at most mild stress in the animals. Only two tests may cause a moderate level of stress for a few minutes.

3. Due to anaesthesia and pain management, the animals should only perceive the initiation of anaesthesia (injection).</td></tr>
<tr><td rowspan="6">What species and numbers of animals are expected to be used? What are the expected severities and the numbers of animals in each severity category (per species)?</td><td>Species[4]</td><td>Estimated total numbers</td><td colspan="4">Estimated numbers per severity</td></tr>
<tr><td></td><td></td><td>Non-recovery</td><td>Mild</td><td>Moderate</td><td>Severe</td></tr>
<tr><td>Mouse (Mus musculus)</td><td>420</td><td>170</td><td>160</td><td>90</td><td>0</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>What will happen to the animals kept alive at the end of the procedure?[5, 6]</td><td colspan="2">Estimated number to be reused</td><td colspan="2">Estimated number to be returned to habitat/husbandry system</td><td colspan="2">Estimated number to be rehomed</td></tr>
<tr><td>Please provide reasons for the planned fate of the animals after the procedure.</td><td colspan="6">The brains of some of the mice will be removed under deep anaesthesia, which results in the death of the animals. All the other animals used in the study will be humanely euthanised in order to collect their brains for further analysis.</td></tr>
<tr><td colspan="7">Application of the Three Rs</td></tr>
<tr><td>1. Replacement
State which non-animal alternatives are available in this field and why they cannot be used for the purposes of the project.</td><td colspan="6">The planned investigations are partially built on findings from cell culture experiments. Other alternatives including brain reaggregate cell cultures and organotypic cultures can only partially reconstruct the complexity of the brain and are therefore not yet sufficient to answer the research questions in this project. Especially behavioural studies rely on using living organisms. We plan to investigate during this project if it is possible to develop a specific neuronal cell line to further perform in-vitro assays.</td></tr>
<tr><td>2. Reduction
Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce the number of animals to be used, and principles used to design studies. Where applicable, describe practices that will be used throughout the project to minimise the number of animals used consistent with scientific objectives. Those practices may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.</td><td colspan="6">The calculation of the required number of mice is based on a pilot experiment and relevant existing literature. The number of animals we plan to use should lead to robust and interpretable data. Individual mice will undergo more than one behavioural test (including breaks), so that a maximum amount of data can be obtained from each animal and the overall number can be reduced.</td></tr>
<tr><td>3. Refinement
Give examples of the specific measures (e.g., increased monitoring, post-operative care, pain</td><td colspan="6">There will be daily contact between mice and staff and training of mice prior to the behaviour tests, to reduce the stress levels during procedures. The animals will be monitored frequently and animal welfare score sheets will be used. This shall ensure that no animal exceeds the pre-determined level of distress. If an animal is found to be experiencing unexpected adverse effects,</td></tr>
</table>

management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms to take up emerging refinement techniques during the lifetime of the project.	they will be killed humanely. If new refinement options become known during the project, they will be applied immediately.
Explain the choice of species and the related life stages.	Mice have long been used in biomedical research and are therefore very well understood. Furthermore, the creation and breeding of genetically modified mice is very advanced and allows investigations down to the molecular level. The aim of the project is the analysis of postnatal development of the brain, therefore mice in an early life stage (3 days after birth) will be used.

Project selected for Retrospective Assessment	Deadline	Contains severe procedures	Uses non-human primates	Other reason X
Explanation of the other reason for retrospective assessment				
National legislation requires a retrospective assessment for projects with the severity category “moderate”.				

RAR

Title (as per Non-technical Project Summary)	Studying the mechanisms of brain development and their role in behavioural disorders		
Reason for Retrospective Assessment ([8])	**Using non-human primates**	**Contains severe procedures**	**Other reason X**
Explain ‘Other reason’	National legislation requires a retrospective assessment for projects with the severity category “moderate”.		
Achievement of objectives			
Explain briefly whether, and to what extent, the objectives set out in the authorised project have been achieved. Provide reasons if objectives have not been attained. Have there been any other significant findings? What benefits have resulted from the work to date, and are further benefits expected? Have the results of this project been disseminated, including where hypotheses are not proven? If so, describe how. If not, indicate how and when results are expected to be publicised.	This project aimed to understand the function of a certain group of regulatory genes in the later stages of the formation of the central nervous system, following birth, especially in context of neurodevelopmental disorders. Researchers reported that the results of the microscopic study of cells and molecular biology experiments has highlighted the importance of one specific gene, in the formation and final development of the synapses – where messages are exchanged inside the brain. These findings will serve as a basis for further research projects, in order to better understand the mechanisms in the formation of the central nervous system and the development of disorders. The results were published in a peer-reviewed journal and preliminary data was presented at international conferences.		

	In another part of the project, it was planned to investigate the loss-of-function of this important gene, especially in relation to autism spectrum disorders. It was planned to carry out behavioural tests with different genetically modified mouse strains. Researchers reported that, unfortunately, it turned out that one of the genetically modified mouse strains was not suitable for the planned experiments. Due to time constraints, it was not possible to carry out those experiments with another mouse strain.

Harms

Species [9]	**Total numbers of animals used**	**Numbers of animals per actual severity**			
		Non-recovery	**Mild**	**Moderate**	**Severe**
Mouse (*Mus musculus*)	121	19	85	17	0

How do numbers of animals used and actual severities compare with those estimated? Where the actual numbers are higher than the estimated numbers, please provide an explanation. Where the actual numbers are lower, please provide an explanation unless that difference is a result of Reduction or Refinement?	The numbers of animals used was lower than estimated. Some experiments had to be skipped due to time constraints and on the other hand due to changes in experimental design. The actual cumulative severities for the mice were the same as estimated.
How does the fate of animals kept alive at the end of the study compare with the estimated fate? Please provide an explanation.	No animal was kept alive at the end of the study. The animals used in this project were killed under anaesthesia at the end of the experiment in order to use the organs and tissue for further investigations.

Any elements that may contribute to further implementation of the Three Rs:

1. Replacement

With the knowledge obtained from this project, have any new approaches that could replace some or all of the use of animals in similar projects been identified/developed (including the development/validation of new *in vitro* or *in silico* techniques)?	In the third part of the project brains of genetically modified mice were analysed. According to the preliminary data, researchers think that it seemed to be possible to continue investigations using cell cultures. As a result, a neuronal cell line – a population of cells maintained in the laboratory that can be used for an extended period of time with the necessary characteristics and functions required – has been developed and used during the development of the project, which could help partially replacing animals, and will also be used in future studies.

2. Reduction

With the knowledge obtained from this project, could the experimental design be improved to	Originally, it was planned to only use male mouse pups for the experiments. It was necessary for the gene expression to be induced one day after birth; however,

enable any further reduction of the use of animals, and if so, how? Provide an explanation where numbers of animals used were lower than those originally estimated.	this was a time point where the determination of the sex of the animal was not possible. This meant that all pups, including females, were treated. Therefore, it was decided to include male and female mice in the protocol. Consequently, the overall number of animals could be reduced. Due to colony management strategies, the use of both sexes and a collaboration with another research group, using the same mouse strains in a different project, all animals bred were used in procedures. Further, the partial replacement of mouse brain experiments also reduced the number of animals used.
3. Refinement	
Provide an explanation where the actual severities were lower than those originally estimated. With the new knowledge obtained from this project, are the animal models used still the most appropriate? Please specify per species/model, where appropriate. List any novel refinements introduced during the project to reduce harm to the animals or to improve their welfare. What are the potential opportunities for further refinement in the future, for example, emerging technologies, techniques, improved welfare assessment methods, earlier endpoints, housing/husbandry measures?	The mouse remains an appropriate model for studying neurodevelopmental disorders, such as autism, which involve the study of social interactions or repetitive and stereotyped behaviours. However, according to the researchers, the method used to inactivate the gene of interest (injection of the hormone) was reviewed for minimising the dose and the stress experienced by the young mice. It was possible to reduce the duration of the treatment time from 4 to 3 days, and to reduce the final concentration of the drug agent, while ensuring a 90% reduction in the expression of the gene of interest. This refinement therefore reduced the number of injections needed while maintaining their effectiveness and increased the well-being of the animals. This procedure is now commonly used by the research group.
4. Other	
How are the findings for further implementation of the Three Rs disseminated?	The improved injection method used to inactivate the gene of interest and the established neuronal cell line will now be included in the next group publication. Furthermore, the group will present these data in the form of posters/communications within the framework of relevant thematic conferences (e.g. FELASA – the Federation of European Laboratory Animal Science Associations).
Additional comments	

This is considered a good quality RAR because:

- **Language is clear, concise and understandable to a layperson;**
- **It is anonymous;**
- **It is explained adequately whether and to what extent, the objectives set out in the authorised project have been achieved;**

- **Reasons for objectives, that have not been attained, are provided;**
- **It is explained why the number of animals is lower than estimated in the application;**
- **New findings that contribute to Three Rs are stated and clearly described;**
- **It shows how the developments in a project can lead to replacement during the development of the project and as a new replacement tool for further studies;**
- **Information on how findings on the Three Rs will be disseminated is provided;**
- **It is explained why the animal model used remains appropriate.**

Example 6 (poor quality)

NTS

Title of the project	Repeated brain trauma in rat and mouse
Duration of project (in months)	60 months
Keywords	Cerebral damage; TBI; CTE
Purpose of project (multiple choices possible)	The effects of diseases, ill-health and other abnormal conditions on humans, animals or plants and how to prevent, diagnose or treat them
Objectives and predicted benefits of the project	
Describe the objectives of the project (for example, addressing certain scientific unknowns, or scientific or clinical needs).	Repeated brain shock is a consequence of many sports, not only in martial sports like boxing, but also, for example, in sports like ice hockey and American football. It has been shown that many athletes who have suffered multiple brain shocks a time after the end of their career have experienced memory disorders, changes in personality and depression. When brains of these athletes were investigated after they died, changes similar to those of Alzheimer's disease were observed in their brain, including the accumulation of the tau protein. In this study we want to establish models of rat and mouse for repeated mild trauma on the head and explore the mechanisms that can cause this chronic brain damage. We will study behavioural changes and changes in the brain by using brain scanning techniques like PET/MR and test a potential medicine in order to develop a treatment.
What are the potential benefits likely to derive from this project? Explain how science could be advanced, or humans, animals or environment may ultimately benefit from the project. Where applicable, differentiate between short-term benefits (within the duration of the project) and long-term benefits (which may accrue after the project is finished).	With these trials, we hope to be able to better understand how unwanted protein deposits occur and how they may be treated. As we will develop the animal model, we will initially focus on how to simulate the disease in rodents, with regard to the extent and frequency of the damage that should be given to produce the same symptoms as seen in human patients.
Predicted harms	
In what procedures will the animals typically be used (for example, injections, surgical procedures)? Indicate the number and duration of these procedures.	The animals will receive brain trauma in a single or in repeated sessions while under anaesthesia. A surgery will be performed to open the skull (craniotomy) and a carefully calibrated weight is dropped on meninges to create a standardised impact comparable with a mild concussion. After surgery, the wound will be closed and the animals will have time to recover. Changes in the brain will be investigated with imaging techniques, using radioactive isotopes (PET) or magnetic resonance (MRI) that can visualise the brain form outside the animal. During these imaging sessions, the animals will be sedated. Behavioural effects, like motor and memory function will also be investigated using a behavioural test called 'Novel object recognition test' in which animals have to recognise novel objects from familiar ones. These tasks do not cause any discomfort, except for small amount of stress during a short training period prior to the actual tests. After a fixed observation period, the animals

<table>
<tr><td></td><td colspan="5">will be humanely killed, in order to study the damage to their brain on an anatomical and biochemical level.</td></tr>
<tr><td>What are the expected impacts/adverse effects on the animals, for example pain, weight loss, inactivity/reduced mobility, stress, abnormal behaviour, and the duration of those effects?</td><td colspan="5">We expect animals to deteriorate in memory and possibly motor function shortly after the brain trauma. In longer experiments, the motor function may deteriorate further as a result of protein accumulation, but the animals will survive up to maximum one month after the injury. Also, animals will experience pain and stress, due to the brain surgery, sedation and training for the behavioural tests.</td></tr>
<tr><td>What species and numbers of animals are expected to be used? What are the expected severities and the numbers of animals in each severity category (per species)?</td><td colspan="5"><table>
<tr><th>Species[4]</th><th>Estimated total numbers</th><th colspan="4">Estimated numbers per severity</th></tr>
<tr><th></th><th></th><th>Non-recovery</th><th>Mild</th><th>Moderate</th><th>Severe</th></tr>
<tr><td>Mice</td><td>550</td><td>0</td><td>0</td><td>550</td><td>0</td></tr>
<tr><td>Rats</td><td>550</td><td>0</td><td>0</td><td>550</td><td>0</td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table></td></tr>
<tr><td>What will happen to the animals kept alive at the end of the procedure?[5, 6]</td><td colspan="2">Estimated number to be reused</td><td colspan="2">Estimated number to be returned to habitat/husbandry system</td><td>Estimated number to be rehomed</td></tr>
<tr><td>Please provide reasons for the planned fate of the animals after the procedure.</td><td colspan="5">After the experiments, the animals will be humanely killed so that their brain tissue can be collected for further analysis of the anatomical and biochemical effects of repeated brain trauma.</td></tr>
<tr><td colspan="6">Application of the Three Rs</td></tr>
<tr><td>1. Replacement
State which non-animal alternatives are available in this field and why they cannot be used for the purposes of the project.</td><td colspan="5">The biological systems involved, like the brain cells, blood circulation and the immune system are so complex that an intact organism is required to study the effects that take place after traumatic brain injuries. The interactions between these systems are so complex that cell cultures cannot closely simulate the course of injury.</td></tr>
<tr><td>2. Reduction
Explain how the numbers of animals for this project were determined. Describe steps that have been taken to reduce the number of animals to be used, and principles used to design studies. Where applicable, describe practices that will be used throughout the project to minimise the number of animals used consistent with scientific objectives. Those practices may include e.g. pilot studies, computer modelling, sharing of tissue and reuse.</td><td colspan="5">We have decreased the number of animals compared to sham-lesioned animal models. Going even lower would make it difficult to be able to ensure statistical differences between the groups due to a certain variability between animals. We are aware that a relatively large number of animals is used for the studies, but a large number of groups is necessary due to the complexity of the procedures. As far as possible, animals are used for multiple parameters (histology, biochemistry, physiology, behaviour) to further reduce the required number of animals.</td></tr>
<tr><td>3. Refinement
Give examples of the specific measures (e.g.,</td><td colspan="5">We have used relevant models of traumatic brain injury for many years and are familiar with their consequences. Animals are handled and treated to</td></tr>
</table>

increased monitoring, post-operative care, pain management, training of animals) to be taken, in relation to the procedures, to minimise welfare costs (harms) to the animals. Describe the mechanisms to take up emerging refinement techniques during the lifetime of the project.	minimise discomfort when monitoring parameters of weight and animal welfare. Strict exclusion criteria are used to minimise animal suffering.
Explain the choice of species and the related life stages.	Mice and rats are the most studied mammals in science and their brains and biology are comparable with the human brain to a large extent. Hence, there is already a lot of knowledge about their neurobiology and this will help to translate findings about traumatic brain injuries to the human situation. Also, protocols for behavioural tasks and brain imaging techniques like MRI and PET are already available and well validated for these species.

Project selected for Retrospective Assessment	Deadline	Contains severe procedures	Uses non-human primates	Other reason X
Explanation of the other reason for retrospective assessment				
Model under development which could contain severe procedures				

RAR

Title (as per Non-Technical Project Summary)	Repeated mild traumatic brain damage in rat and mouse		
Reason for Retrospective Assessment ([8])	**Using non-human primates**	**Contains severe procedures**	**Other reason** **X**
Explain 'Other reason'	Model under development which could contain severe procedures		
Achievement of objectives			
Explain briefly whether, and to what extent, the objectives set out in the authorised project have been achieved. Provide reasons if objectives have not been attained. Have there been any other significant findings? What benefits have resulted from the work to date, and are further benefits expected? Have the results of this project been disseminated, including where hypotheses are not proven? If so, describe how. If not, indicate how and when results are expected to be publicised.	The objective has only been partially achieved. The results obtained will feed into further studies. No. No. No, but the results were included in a student report.		

Harms

Species (9)	Total numbers of animals used	Numbers of animals per actual severity			
		Non-recovery	Mild	Moderate	Severe
Mice	24	0	15	9	0
Rats	0	0	0	0	0

How do numbers of animals used and actual severities compare with those estimated? Where the actual numbers are higher than the estimated numbers, please provide an explanation. Where the actual numbers are lower, please provide an explanation unless that difference is a result of Reduction or Refinement?	University of Europia justified the experiments as follows: 15 animals were exposed to a single mild traumatic brain injury and no neurological signs of disability were found. Post-mortem analysis of the brain showed no evidence of brain injury. As the animals were studied for a short-time and showed no signs of injury, the severity is considered to be mild. Less animals were used because the researcher group left the institution.
How does the fate of animals kept alive at the end of the study compare with the estimated fate? Please provide an explanation.	Does not apply

Any elements that may contribute to further implementation of the Three Rs:

1. Replacement

With the knowledge obtained from this project, have any new approaches that could replace some or all of the use of animals in similar projects been identified/developed (including the development/validation of new *in vitro* or *in silico* techniques)?	No.

2. Reduction

With the knowledge obtained from this project, could the experimental design be improved to enable any further reduction of the use of animals, and if so, how? Provide an explanation where numbers of animals used were lower than those originally estimated.	No. The investigator and staff changed their place of work.

3. Refinement	
Provide an explanation where the actual severities were lower than those originally estimated. With the new knowledge obtained from this project, are the animal models used still the most appropriate? Please specify per species/model, where appropriate. List any novel refinements introduced during the project to reduce harm to the animals or to improve their welfare. What are the potential opportunities for further refinement in the future, for example, emerging technologies, techniques, improved welfare assessment methods, earlier endpoints, housing/husbandry measures?	(no information provided) (no information provided) They monitored parameters of weight for animal welfare (no information provided)
4. Other	
How are the findings for further implementation of the Three Rs disseminated?	(no information provided)
Additional comments	(no information provided)

This is considered a poor quality RAR because:

- **Language is not clear, concise and understandable to a layperson (i.e. neuroscientific terms like traumatic damage and post-mortem could be simplified);**
- **It is not anonymous (i.e. institution is indicated in the harms section);**
- **Although under certain circumstances it is acceptable to answer 'Not applicable/No information provided', in this case important information is lacking. For example, the partial attainment of objectives and the dissemination of the results are not elucidated, also no comparison is provided with the estimated severities and /or timeline results will be published was not clearly described;**
- **Harms section. Although the harms for the animals were lower than expected, this is not a justification for the experiments that were carried out. The harms of the experiments should be justified by the benefits (scientific, human, animal or environmental) provided by their results or explanation provided;**
- **The harms section in the RAR simply reports the harms as given by the researcher, instead it should reflect the assessment carried out by the Competent Authority on the basis of the information provided by the project authorisation holder;**
- **In the 'Refinement' section, it is not explained why the actual severity was lower than expected or 'whether the animal models used are still the most appropriate';**
- **In addition, it contains contradictory information. For example, in the 'achievement of objectives' section it is said that; "no benefits have resulted from the work". And yet, in the same section, it is said that; "The results obtained will feed into further studies." and; "the results were included in a student's report;**
- **Information is provided in the wrong sections, for example the phrase: "The investigator and staff changed their place of work." is presented in the reduction section, but this is not a reduction measure;**

- **It contains misleading information. Monitoring weight and welfare parameters is presented as being a novel refinement measure.**

Appendix III – List of Member States where NTS update with RAR is mandatory (status 2022)

AT Austria

BE Belgium

BG Bulgaria

CY Cyprus

DE Germany

EL Greece

FI Finland

HR Hungary

LT Lithuania

MT Malta

NL The Netherlands

PL Poland

PT Portugal

RO Romania

SE Sweden

SI Slovenia

SK Slovakia

GETTING IN TOUCH WITH THE EU

In person

All over the European Union there are hundreds of Europe Direct information centres. You can find the address of the centre nearest you at: https://europa.eu/european-union/contact_en

On the phone or by email

Europe Direct is a service that answers your questions about the European Union. You can contact this service:

— by freephone: 00 800 6 7 8 9 10 11 (certain operators may charge for these calls),
— at the following standard number: +32 22999696 or
— by email via: https://europa.eu/european-union/contact_en

Finding information about the EU

Online

Information about the European Union in all the official languages of the EU is available on the Europa website at: https://europa.eu/european-union/index_en

EU publications

You can download or order free and priced EU publications at: https://op.europa.eu/en/publications. Multiple copies of free publications may be obtained by contacting Europe Direct or your local information centre (see https://europa.eu/european-union/contact_en).

EU law and related documents

For access to legal information from the EU, including all EU law since 1951 in all the official language versions, go to EUR-Lex at: https://eur-lex.europa.eu

Open data from the EU

The EU Open Data Portal (https://data.europa.eu/en) provides access to datasets from the EU. Data can be downloaded and reused for free, for both commercial and non-commercial purposes.

KH-02-24-265-EN-N

Publications Office
of the European Union