Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	Provide the title of the project.	Define optimal injection strategy of an antisense oligonucleotide (ASO) for Angelman Syndrome

2 Categories

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

- ☐ Basic research
- X Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Angelman Syndrome (AS) is a rare genetic neurodevelopmental disorder (approximately 1 in 15,000 people), characterized by developmental delay, small head and brain, severe speech impairment and

sleeping disorders. The delayed development only becomes apparent at around 6-12 months of age. A large number of patients have seizures and/or develop scoliosis. Most children have a happy excitable personality with frequent smiling and hand flapping movements. The sleeping disorders and hyperexcitability can improve with age, but AS patients need lifelong 24h care. The life expectancy appears to be normal, or maybe slightly reduced (max reduction of 10 years) (Margolis et al., 2015). AS is caused by a defect in the maternal copy of the gene ubiquitin protein ligase E3A (UBE3A). UBE3A normally exhibits many functions besides ubiquitin transfer, like helping to degrade misfolded proteins, regulation of the circadian clock, coactivator in transcriptional activation of the progesterone receptor and, importantly, regulation of synaptic development and function. Mouse studies have shown that specifically in the brain, the paternal copy of the gene is silenced in neurons by a non-coding antisense transcript (Ube3-ATS) while it is biallelically expressed in other tissues. This brain-specific imprinting of UBE3A is presumably found in humans as well. The majority of human AS cases (70-80%) is caused by deletion of a part of the maternal chromosome 15 (15q11-q13); these patients show the most severe phenotype. Other molecular mechanisms include intragenic mutations, paternal uniparental disomy (2 copies of the paternal gene, none from the mother) or imprinting defects within chromosome 15q11-13 that alter the expression of the maternal copy of the gene (Margolis et al., 2015).
There is no specific therapy available to treat these patients, although some of the symptoms of the disease can be treated. Often AS patients are treated with anti-epileptics to reduce the seizures, and some are on limited sleep medication and/or on Ritalin. A more targeted treatment aiming to restore the lost expression of the parental UBE3A gene and as a consequence of that hopefully restoring the cognitive capacity, could potentially be a life-changer for these people and their care-takers.

Rodent models for AS
Mouse models were generated by targeted inactivation of maternal UBE3A. Upon inheritance of the maternal UBE3A deletion (with an intact paternal copy), mice display many features of the Angelman syndrome, like impaired motor function, seizures and reduced capacity for context-dependent and spatial learning (Margolis et al., 2015).
From these mice, a lot of information has been generated regarding molecular and cellular events underlying this syndrome. Loss of UBE3A in mice has impact on the expression levels in the brain of several genes involved in synapse development and synaptic function (Greer et al., 2010). Moreover, histone deacetylases (HDAC) 1 and 2 were overexpressed in AS-mice, already during embryonic development (Jamal et al., 2017). HDACs regulate expression of many genes through chromatin remodeling, so the impact of overexpression of these two genes on the development of the brain may be huge, and therefore normalization of the expression levels may have a positive impact on the functionality of the brains of AS patients.
Recently a novel model of AS is developed in rats by deleting the entire UBE3A gene (Berg et al., 2020). In mouse models, only a single exon is often deleted, with the existence of three variants leading to inconsistency in results and the likelihood that not all mouse studies truly showed the full spectrum of AS. The deletion of the entire gene resulted in a comprehensive rodent model which shows the full spectrum of both functional phenotypes (reduced vocalizations, delayed reflex developments and motor deficits) and anatomical alterations (decreased brain volume). This last point will be challenging to improve with a novel therapy though the quality of life of patients will be improved when the functional behavioral, in relation to AS, could be improved.

Possible treatments
A potential treatment to normalize HDAC1/2 expression levels would be possible by using Simvastatin, an HMG CoA (3-Hydroxy-3-methylglutaryl-CoA) reductase enzyme repressor, commonly used as cholesterol-lowering drug. This blood-brain barrier passing drug, is known to inhibit expression of HDAC1 and 2, apart from its cholesterol-reducing activity (Lin et al., 2008). Treatment of AS mice with Simvastatin reduced the HDAC1/2 levels, increased the brain derived neurotrophic factor (BDNF) expression and significantly improved the cognitive functions of the mice (Kumar et al., 2019). These are very promising results, but translation from mouse to man is particularly difficult for neurodevelopmental disorders (Ottenhof et al., 2020) and this requires further research.

Another therapeutic approach is the induction of reactivation of the parental copy of the gene in the brain, this can be achieved by an oligonucleotide targeting the inactivating antisense ribonucleic acid (RNA). In this way it prevents the interaction between the antisense RNA and the intact paternal copy of the gene, thereby reverting the expression of the UBE3A gene. Mild amelioration of the memory impairment was observed in AS mice upon treatment with an oligonucleotide targeting the antisense UBE3A RNA (Meng et al., 2013), even when treated during adulthood.
Very few clinical trials in AS patients have been performed. Up to now (September 2020) 5 different drugs have been tested in clinical trials: Betaine/folic acid gave no improvement and the results of other four drug treatments: levodopa, minocycline, OV-101 (gamma-aminobutyric acid (GABA) agonist) and GTX-102 (antisense oligo) so far have not been made publicly available.

In this project, we focus on the reactivation of the parental copy of UBE3A. Two clinical trials will start this year using an antisense oligonucleotide.

RO7248824 is a 20-mer antisense oligonucleotide (ASO) that reactivates expression of UBE3A protein in neurons of AS patients (unpublished data).
RO7248824 was identified by an in-vitro locked nucleic acid (LNA) screening platform using human induced pluripotent stem cell (hiPSC) neurons derived from Angelman Syndrome subjects. RO7248824 produced a dose dependent reduction of the UBE3A antisense (UBE3A-ATS) with median IC50 (inhibitor concentration that reduces the response by half) of 7 nM and a dose dependent increase in the UBE3A sense with median EC50 (drug concentration that gives half-maximal response) of 35 nM following a 5-day incubation period.

RO7248824 showed consistent dose-dependent reductions of UBE3A-ATS RNA and dose-dependent increases in UBE3A sense RNA and UBE3A protein in iPSC neurons derived from both diseased and non-diseased human neurons, as well as cynomolgus wildtype (WT) neurons.

The in-vitro results confirm that the concept of ASO-mediated Ube3a-ATS targeting to upregulate Ube3a mRNA and UBE3A protein, as previously shown in mice (Meng et al., 2015), is translatable to human and cynomolgus neurons in a disease and non-disease context. This enabled further assessment of pharmacodynamics properties of RO7248824 *in-vivo* in nonhuman primates.

RO7248824 pharmacodynamics (PD) was studied in two cynomolgus macaque studies following intrathecal (IT) injections, the intended clinical administration route. Pharmacodynamic responses were measured in various regions of the brain by analysing: 1) UBE3A-ATS RNA, 2) UBE3A sense mRNA and 3) UBE3A protein.

In normal macaques, UBE3A is expressed from the maternal allele. Once the UBE3A paternal allele is unsilenced, UBE3A mRNA is expected to be expressed from both alleles and protein expression should increase up to 200% upon treatment, enabling PD analysis in non-diseased macaques.

Single- (and repeat-) Dose PK/PD Study in Macaques

RO7248824 was administered once or twice by IT injection to cynomolgus macaques. A single dose of 24 mg RO7248824 in macaques resulted in long-lasting pharmacodynamic effects in several brain regions. Those effects were *UBE3A-ATS* reduction, *UBE3A*-sense mRNA and UBE3A protein induction and were sustained over at least 85 days (end of study), compared to vehicle-treated macaques.

Best PD effects were observed in cortex regions (target tissues), hippocampus and cerebellum, while the PD effects were lower in midbrain, medulla and pons due to lower tissue LNA uptake.

An 8-week toxicity study was conducted in male and female cynomolgus macaques with a recovery period of 8 weeks. RO7248824 was administered IT at 4, 14, or 30 mg/animal doses once every 4 weeks (on Days 1, 29, and 57 for a total of 3 doses).

Repeated dosing of RO7248824 in macaques resulted in dose-dependent reduction in UBE3A-ATS, as well as dose-dependent increases in UBE3A-sense transcript and UBE3A protein at Week 9 (Day 64). In the 30-mg dose group the median cortical level of UBE3A-ATS was 95% lower, UBE3A-sense transcript 209%

higher and UBE3A protein 179% higher, compared to vehicle-treated macaques. At Week 17 (Day 120), reduction in UBE3A-ATS as well as elevations in UBE3A-sense and UBE3A protein levels were still measurable in the 14- and 30-mg dose groups. Strongest effects were observed in cortical regions for all three PD parameters as well as in spinal cord for UBE3A-ATS, hippocampus for UBE3-sense and dorsal striatum and hippocampus for UBE3A protein.

Positron emission tomography-computed tomography (PET-CT) studies in healthy volunteers are planned, ungated study from the non-human primate (NHP) study, to be performed with a sub-pharmacological dose of ^{89}Zr-labeled RO7248824 and 4 mg RO7248824 (low dose in good laboratory practice (GLP) toxicity) administered via IT injection. We learned that IT administration procedures can be quite complex and volumes of administration and flushing volumes can impact distribution of our compound into the brain as we have learned from our previous study in NHPs that with an administration of 1 ml dose and 0.25 ml flushing volume the concentration in the brain was much lower compared to flushing with 1.5 ml.
To better understand the impact of dose administration and translation from NHPs to human with RO7248824 we would like to test several conditions in a macaque experiment. The data will refine the pharmacokinetic/pharmacodynamic (PK/PD) model that is used to choose the optimal conditions for the upcoming human trial and to verify the translation from macaque to human. The data will inform us what the best IT administration procedure is for future studies.

Literature:

- Margolis et al.; Angelman Syndrome. Neurotherapeutics. (2015)
- Greer et al.; The Angelman Syndrome protein Ube3A regulates synapse development by ubiquitinating arc. (2010)
- Jamal et al.; Rescue of altered HDAC activity recovers behavioural abnormalities in a mouse model of Angelman syndrome. Neurobiol. Dis. (2017)
- Berg et al.; Translational outcomes in a full gene deletion of ubiquitin protein ligase E3A rat model of Angelman syndrome. Transl Psychiatry.(2020)
- Lin et al.; Statins increase p21 through inhibition of histone deacetylase activity and release of promoter-associated HDAC1/2. Cancer Res. (2008)
- Kumar et al.; Simvastatin Restores HDAC1/2 Activity and Improves Behavioral Deficits in Angelman Syndrome Model Mouse. Front Mol Neurosci. (2019)
- Ottenhof et al.; Considerations for Clinical Therapeutic Development of Statins for Neurodevelopmental Disorders. eNeuro. (2020)
- Meng et al.; Truncation of Ube3a-ATS unsilences paternal Ube3a and ameliorates behavioral defects in the Angelman syndrome mouse model. PLoS Genet. (2013)
- Meng et al., Towards a therapy for Angelman syndrome by targeting a long non-coding RNA. Nature. (2015)

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The goal is to determine which IT administration method needs to be used in NHPs by IT injecting ASOs into the brains of cynomolgus macaques, and using PET-CT, fused with a previous obtained MRI, to determine the uptake pattern and measurements in cerebrospinal fluid (CSF) and blood to monitor the compound distribution in the brain at several timepoints post injection. The goal is to find the administration technique that results in the highest drug concentration in the brain and is feasible to use in humans. The macaque data will later be compared to the data from the healthy human volunteers to learn more about the translatability from macaque to humans. The second goal is to be able to generate data that correlate ASO concentrations in CSF to the concentrations in human brain.
These goals are achievable, because we can measure differences in drug distribution to determine the best method. We will apply the ASO in different ways: 1) by injecting the same amount of ASO in different

injection volumes and with or without flushing after injection 1) by replacing part of the CSF with artificial CSF containing ASO, 2), and measure drug concentration. Based on those measurements we can refine our PK/PD model. We scale from NHP to human by a factor of 10, since the CSF volume in humans is 10x higher than in NHPs. The ultimate goal is to generate a safe, effective and tolerable treatment for AS patients.

Feasibility of the project:
For various reasons we believe that our research objectives can be achieved:

1. The rationale and objective is supported by a relevant body of (recent) literature and (ongoing) *in vitro*, *ex vivo* and *in vivo* experiments, and we have ample experience with the proposed research strategies (Rotaru et al., Neuroscience, 2020, Feb 21:S0306-4522(20)30103-2).
2. The high quality of our work is confirmed by the publications in leading international scientific journals (e.g Dijkman et al., Nature Medicine (2019), van der Aart et al., Mol Imaging Biol (2019), Sterck et al., Am J Primatol (2019).).
3. We have an excellent work environment with experienced persons, state-of-the-art techniques (e.g. PET-CT) and animal models; and actively collaborate with world leading experts in academia and industry.

3.3 Relevance

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

Angelman Syndrome is a debilitating neurodevelopmental disorder in which intellectual functions, social communication, cognition and behaviour are separate impaired which has a substantial impact on the quality of life of patients with e.g. sleep problems, seizures, gastrointestinal problems and disruptive/aggressive behaviours. At present there is no specific treatment only supportive treatment available for AS.
The project proposed in here is specifically designed as a tool to develop protocols for ASO administration in the brain which could, in the end, restore the function of the impaired gene. Drug delivery to the brain is challenging due to the blood-brain barrier (BBB). By injecting it into the CSF, distribution to the ependymal surface of the brain is at least ensured but further distribution into the deeper parachyma is unknown though essential for a successful treatment approach. Nevertheless, a great interpatient variability in spread is observed in literature. Convective forces generated in the CSF by the IT injection itself can increase the spread of the drug in the brain. Other convective forces which can have an effect on the distribution are CSF turnover, cardiac motion, respiratory thoracic motion and body movement. To be able to translate the results to the clinic a species with comparable behaviour on those points should be used (Hocking et al. (2004), Sullivan et al. (2020)). Nevertheless, still experiments in humans are required to assess the translatability and to see if the conclusions drawn from rodents and NHPs are similar for humans too.
In here, we will analyse the compound distribution in healthy macaque brains using different application methods (determine optimal application volume resulting in highest drug concentration in the brain and testing whether replacement of macaque CSF with artificial CSF containing the compound has impact on the tissue distribution of the compound). So if increasing the pressure is causing for convective forces which will increase the rostral delivery of the drug or instead of increasing the pressure, keep the pressure similar as CSF distribution is homogenous under homeostatic conditions. The results from this experiment will be used to design a protocol for optimal administration of this compound in humans, resulting in pharmacological brain exposure and best benefit risk ratio. As the risk is on one hand that we dose too low and the patient will not benefit enough from the treatment. On the other hand, the risk of dosing to high is low and unlikely as we are covered by the NHP toxicity studies already performed. This enhances the chance of a positive outcome for the clinical trials with AS patients. As mentioned, the treatment for AS patients up to now consists only of suppression of the symptoms. With this new approach, a more targeted treatment could be developed, which could result in alleviation of the physical problems, improving the mental skills and quality of life of these people.

Literature

- Hocking et al.; Intrathecal drug spread. BJA. (2004)

- Sullivan et al.; Convective forces increase rostral delivery of intrathecal radiotracers and antisense oligonucleotides in the cynomolgus monkey nervous system. J Transl Med. (2020)

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

The overall strategy is to develop an optimized injection method suitable for application of ASO RO7248824 in NHPs and ultimately in Angelman Syndrome patients. For this, macaque experiments are necessary as the brain anatomy and physiology resembles the human brain best. Macaques will be injected with labeled and unlabeled ASOs in different volumes, using different application methods to determine which procedure is needed to obtain the desired tissue distribution.
The first macaque experiment will result in the optimal flushing volume of dosing the compound (low or high flushing volume) and if using a high volume without flushing, results in the same brain exposure.
The second part of the experiment will look into the influence of CSF withdrawal before drug administration. This second part is using the same macaques that have been used in the first part after the Zr-89 is decayed (half-life is 78.4h, around 10 half-life's are used which is approximately 30 days).
PET-CT, blood and CSF measurements will be performed to determine the effect of the procedure on the distribution of the ASO.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

Macaques will be injected with an oligonucleotide targeting the antisense UBE3A transcript that inactivates the paternal copy of the gene. Before injection of the compound, information is needed about the anatomy of the brain and the volume of CSF for each individual animal. Therefore, the experiment will start by performing an MRI of the brain of all macaques. MRIs will be performed on a different site/facility. In this way the soft tissue anatomy of the brain of each macaque is known and can be used for fusion with the PET-CT to combine functional imaging with anatomical imaging. In addition, the MRI is important to be able to determine the volume of CSF, which differs between both humans (140-270 ml) and macaques (9-15 ml). After recovery from the transport to the MRI in their home cage, macaques will be IT injected; by lumbar puncture when placed in left lateral recumbence, in the treatment room of the BPRC. This IT injection method is comparable to humans in which the person is sitting or lying on his side with the back bended. In all cases placement of the needle will be verified by the presence of CSF at the needle hub. They will be injected with unlabeled ASO and hot ^{89}Zr-labeled compound (RO7317427-001-002). Combining unlabeled ASO with labeled compound is the standard procedure to increase the specific concentration of the compound. It is unknown whether labeling with Zr-89 will influence the pharmacokinetics of the compound but labeling with Zr-89 is done with other drugs without any impact on the biodistribution.
PET-CT will be performed at regular time points after injection, and blood and CSF samples will be collected to compare distribution of parent compound and radio-labeled compound. Compound concentrations in blood and CSF will be determined at the same timepoints as when a PET-CT is performed.

For the two experiment the same macaques will be used, as in this way no second MRI is necessary and the variation stays the same. The results of the PK/PD of the first study can be used to determine alterations in this pattern for the second study and vice versa. In addition, otherwise more animals and procedures are necessary. The discomfort of each experiment is considered moderate but the total experienced discomfort of the experiments will not exceed this level. Between the two experiments a wash-out period of 4 weeks will be taken into account.
The first experiment will be performed using a single dose of compound comparing different flushing volumes, or using a high volume without flushing, to determine the optimal flushing volume. In the second experiment we test the influence of CSF withdrawal on drug distribution. We compare distribution with or without CSF withdrawal or using no withdrawal and a high volume.

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

In the first part of the study the optimal flushing volume is determined, in the second part of the study (that will be performed in the same macaques), the impact of CSF withdrawal before drug administration method is investigated.
The milestones for this project are:
1) obtain optimal flushing volume to induce the best tissue distribution
2) obtain information whether it is necessary to withdraw macaque CSF
3) generate a protocol suitable for NHP and human application

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

Serial number	Type of animal procedure
1	Testing of human/cynomolgus macaque UBE3A oligopeptides in a cynomolgus macaque model for Angelman Syndrome (AS).
2	
3	
4	
5	
6	
7	
8	
9	
10	

Appendix
Description animal procedures

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

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	[redacted]
1.2	Provide the name of the licenced establishment.	Biomedical Primate Research Centre
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 1 Type of animal procedure: Testing of human/cynomolgus macaque UBE3A oligopeptides in a cynomolgus macaque model for Angelman Syndrome (AS)

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

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

In this project, we focus on the reactivation of the parental copy of UBE3A. The goal is to determine which intrathecal (IT) administration needs to be used which results in the highest drug concentration in the brain and is feasible to use in humans.

For this purpose, macaques will be injected with an oligonucleotide targeting the antisense UBE3A transcript that inactivates the paternal copy of the gene. For reactivation of UBE3A, expression of the paternal allele is necessary. To achieve this, we need to get rid of the antisense transcript that inactivates the paternal allele.

Before injection of the compound, information is needed about the soft tissue anatomy of the brain and the volume of cerebrospinal fluid (CSF) for each individual animal. This will be obtained via an MRI, performed at a different facility/site. For this the animals will be transported awake in a dedicated transport vehicle. The animals will be trained to voluntary enter the transport box to reduce the amount of discomfort as much as possible. The MRI will be obtained under general anesthesia and will not exceed the 2hr. After waking up, the animal will be transported back to the BPRC.

MRI is an imaging technique to visualize soft tissue with high detail, as the anatomy of every macaque (and human) slightly differs it essential to determine this for each animal specifically to be able to relate the

distribution of the compound in the brain. In addition, there is also variation in the amount of CSF volume between macaques (9-15 ml) and humans (140-270 ml). To be able to determine if variations in results appear due to the percentage of CSF that is altered with the injection this needs to be determined on forehand and based on this the animals could be spread over the groups.
After the MRI (and a recovery period), the macaque will be injected, at the Institutes' facilities, with unlabeled antisense oligonucleotide (ASO) and ^{89}Zr-labeled compound (RO7317427-001-002). A PET-CT will be performed on site at regular time points after injection and blood and CSF samples will be collected to compare distribution of parent compound and radio-labeled compound. Compound concentrations in blood and CSF will be determined at the same timepoints as when a PET-CT is performed.

With the PET-CT in a minimal invasive way the distribution of the drug over time could be followed besides the more general information obtained from the blood and CSF in a PK/PD study. In addition, the image data could confirm the injection to be single-compartmental in the IT space and not multi-compartmental or only partially in the IT space. Even if the veterinarian assesses the injection to be successful. Which makes the outcomes of the study more reliable.

Both blood and CSF will be obtained for the PK/PD of the drug as the drug is injected in the CSF and it is known that this will be distributed to both the brain and the blood. Which percentage will enter the bloodstream could be influenced by the convective forces of the injection. In this way we know for sure that we do not miss anything and will a full spectrum of information.

Two experiments will be performed with a washout period of 4 weeks. The first experiment is to determine the optimal flushing volume by using a single dose of compound comparing different flushing volumes, or using a high volume without flushing. In the second experiment we test the influence of CSF withdrawal on drug distribution. We compare distribution with or without CSF withdrawal or using no withdrawal and a high volume.

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

The test compound will be given once via an IT injection in the distal lumbar spine via the procedure as described in Turner et al., (2013). The BPRC does not have a SOP for the procedure but Vetcare is very experienced with this procedure. IT administration of test substances requires considerable technical skill and in-depth knowledge of anesthesia and spinal cord and vertebral column anatomy. These techniques should be performed only by well-trained personnel and Vetcare has those personnel (see OJT forms of those personnel). By performing the procedure as described by Turner et al. no additional analgesia is necessary as the used anesthetics will provide sufficient analgesia post-procedure for the time discomfort is expected. The injected drug will be delivered on body temperature. A digital temperature-controlled heating bath will be used to deliver it at the right temperature.

Blood (routine procedure described in detail in VOK003) and CSF are taken at multiple time points on the day of injection and subsequently at regular time intervals for PK/PD analysis. In general, blood volumes to be taken will not exceed a maximum of 1% of the body weight per 4 weeks (and 0.7% max per bleeding, anticipated is a volume of 0.5 ml). A CSF volume of 0.2 ml will be sampled which is in general physiological safe (only a couple of percent from the total, assuming a minimal CSF volume of 9 ml (Sullivan et al. (2020)). At those same timepoints a PET-CT will be obtained to follow in a minimal-invasive way the distribution of the test compound over time. During the PET-CT, physiological parameters of the macaques will be closely monitored e.g heartbeat, respiratory frequency, saturation (SpO_2%) and rectal body temperature.

RO7248824, the test compound, has been tested up to 30 mg in cynomolgus toxicity studies, using an IT injection, without any signs of toxicity. The planned study in cynomolgus macaques will help us to refine our modelling.

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

The number of macaques per test group will be determined on the basis of experience gained in *in vivo* experiments in rodents and macaques. It is expected that a group size of 3 macaques will suffice for the determination of the optimal IT administration method needed.

B. The animals

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

Species: Cynomolgus macaques (*Macaca fascicularis*). Both rhesus macaques (*Macaca mulatta*) and Cynomolgus macaques are highly suitable to use for neurological research due to their comparability with humans. In this experiment we would prefer cynomolgus macaques as the previous experiments were also performed in this species (unpublished data). No gender differences were observed in the pharmacokinetic study so both sexes could be used. However, it is preferable to use one sex to diminish variability.

Although the Angelman syndrome (AS) becomes clinical apparent at around 6-12 months of age in humans, we do not see the necessity to replicate this life-stage for non-human primates too, as the goal of this project is not directly disease related but more model refinement related.

Origin: all macaques are purpose bred. They are either bred at our institute or obtained from a certified supplier.

Amount: in this appendix two experiments will be performed with 3 groups of 3 animals each, resulting in a total group of 9 macaques. Based on previous experiments with PK/PD studies this is sufficient to obtain reliable results.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

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

Macaques to be used in this project might have been used in previous studies, provided that this did not had any influence on the normal anatomy and physiology of the brain or on related processes like atherosclerosis or diabetes. Given the long lifespan of this species, re-use will take place in the legal framework described in art. 1 of the law on animal testing.
In addition, the animals will be re-used within this protocol for the two separate experiments. This is acceptable as the experienced discomfort of both experiments is considered to be moderate taken into account a wash-out period of 4 weeks. Nevertheless, the cumulative discomfort of both experiments together is judged as moderate.

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

X No

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

D. Replacement, reduction, refinement

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

<u>Replacement</u>

The goal is to find the administration technique that results in the highest drug concentration in the brain and is feasible to use in humans. The macaque data will later be compared to the data from the healthy human volunteers to learn more about the translatability from macaque to humans. The second goal is to be able to generate data that correlate ASO concentrations in CSF to the concentrations in human brain. The brain is a complex organ, tightly controlled by the central nervous system and involved in many processes and regulates multiple organs. Additionally, the anatomy and blood brain barrier are highly specific. For this we do not think that rodents are an option, due to the different anatomy and related much lower CSF volumes they are not believed to give us data that can be translated to humans. Besides the differences in volume also the impact of the convective forces will influence the distribution; those forces are CSF turnover, cardia motion, respiratory thoracic motion and body movement. To be able to translate the results to the clinic, taken those possible variables into account, a species with comparable behaviour is required.
The ASO tested in this project is currently already being tested in children with AS in a phase 1, multicentre trial to examine the effects of the drug on the body and to measure alterations in AS symptoms. Nevertheless, testing the most optimal delivery method cannot be checked in those children too as the amount of sedations under general anaesthesia will likely influence their quality of life even further and with this the results of this trial.

For similar reasons, current alternative methods, such as cell culture, will only provide limited information about what happens in a whole living animal. Therefore, the use of a non-human primate model for studying the distribution of the test compound after IT injection in the CSF and brain is indispensable.

Reduction
Proper experimental design allows the optimal number of animals to be used in this project in combined with data obtained in previous (pilot) experiments.

Refinement
We use outbred macaques which are excellent models for studying our Research aims. Animals are kept in social groups and are housed in enriched cages. Improvements to procedures which minimize pain and improve animal welfare are included. Anesthetics are used when deemed necessary, and analgesics are given as precaution or as necessity afterwards. Also, macaques will be euthanized when they reach their human endpoint (see below) before the end of the study.
In addition, more specific for this protocol, we use PET-CT as minimal invasive imaging technique which allows us to obtain longitudinal information about the exact distribution of the drug instead of only a general overview obtained with CSF or blood. Besides this, it is an additional control to assess the IT injection.
Other refinement is the use of the same animals for both experiments in this project by which only one MRI at an external location needs to be obtained.

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

Animals will be socially housed with a socially compatible animal. Additionally, animals are trained with positive reinforcement training.
During the study animals will be observed twice daily by qualified and competent animal caretakers, clinical symptoms will be scored using a well-established clinical scoring list. Should changes occur in behaviour, appetite or stool then a veterinarian will be informed and appropriate measures will be implemented. On the basis of the scoring system a clinical human endpoint is defined (see section J). When this endpoint is (unexpectedly) reached, the animal will be immediately euthanized and a full necropsy will be performed to determine the cause of clinical disease. All procedures will be performed under sedation. On every time point where a procedure is performed the animal will be weighed and closely examined for clinical condition.

Repetition and duplication

E. Repetition

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

Not applicable

Accommodation and care

F. Accommodation and care

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

X No

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

G. Location where the animals procedures are performed

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

X No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

The procedures will be performed on fully sedated animals. Pain relieve will be applied if there are any circumstances that indicate that pain can reasonably be expected to occur or animals show signs of illness indicative of pain. Analgesics known not to interfere with the experiment will be used.

I. Other aspects compromising the welfare of the animals

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

1. Discomfort due to the injection of the test compound
2. Stress because of sedation and recovery
3. Stress due to transport for obtaining an MRI to an external licensed institute.
4. Increased intercranial pressure

Explain why these effects may emerge.

1. The test compound has been tested till a dose at least 5 times higher than is planned in here with only transient and non-adverse clinical observations. Nevertheless, when the compound is applied via an IT injection, this can cause local pain and/or irritation.

2. The animals will be repeatedly sedated for procedures. Nausea and disorientation can sometimes be observed during recovery from the sedation.
3. Transport takes animals out of their environment which is stressful.
4. Although slowly, an additional amount of volume is injected in the brain.

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

1. Animals will be sedated for delivery of the test compound. Recovery of the animals will be monitored and the veterinarian will be consulted in case of problems/neurological alterations.
2. The side-effects of sedation cannot be antagonised. However, to prevent nausea and aspiration the animals will fast before sedation. Recovery of the animals will be monitored and the veterinarian will be consulted in case of problems.
3. The animals will be back within a couple of hours and will not stay in the external (licensed) institute. In addition, animals will be trained to voluntary enter the transport box.
4. The volume is injected as slowly as possible and heartbeat and blood pressure are continuously monitored. When an alteration is seen during injection, the injection is paused till the values returned back to the starting position.

J. Humane endpoints

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

☐ No > Continue with question K.

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

Based on the type of experiment a human endpoint is not expected. Though when neurological symptoms are observed (not expected but e.g. due meningitis or CSF leakage), directly the veterinarian will be consulted and appropriate action will be taken.

Indicate the likely incidence.

Based on experience, we anticipate a drop-out rate of <1%.

K. Classification of severity of procedures

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

The amount of discomfort for each experiment is judged as moderate, the total amount of discomfort is also estimated as moderate taken a wash-out period of 4 weeks into account. The discomfort is mainly caused by transport to another facility for the MRI, the sedation needed for collection of blood, CSF sampling and performing PET-CTs.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

X No

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

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

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

☐ Yes

Format
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	Bepalen van de optimale injectie strategie voor een antisense oligonucleotide voor Angelman Syndroom
1.2	Looptijd van het project	01/12/2020 – 30/11/2022
1.3	Trefwoorden (maximaal 5)	Angelman Syndroom, niet-humane primaten, PET-CTs

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☐ Fundamenteel onderzoek
- X Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1	Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)	Angelman Syndroom (AS) is een zeldzame aangeboren genetische neurologische aandoening die een ontwikkelingsachterstand veroorzaakt in combinatie met ernstige spraakstoornissen, een kleinere schedel en slaapstoornissen; vaak in combinatie met epilepsie. Er is voor kinderen met deze aandoening geen specifieke behandeling beschikbaar. AS wordt veroorzaakt door een defect in het ubiquitine eiwit ligase E3A (UBE3A). Het uiteindelijke doel van dit onderzoek is om de functie van dit eiwit te herstellen en hopelijk daarmee de capaciteit en kwaliteit van leven van deze patiënten te verbeteren. In dit onderzoek wordt onderzocht met welke injectietechniek een zo hoog mogelijke concentratie van een specifiek stof, die UBE3A weer kan activeren, in het brein wordt verkregen. Dit wordt uitgezocht met behulp van 2

		experimenten. Door het inspuiten van vloeistof zal tijdelijk de druk in de hersenen verhogen. In het eerste experiment wordt gekeken of deze druk van invloed is op de hoeveelheid teststof die in het brein terecht komt. Dus wanneer dezelfde hoeveelheid teststof opgelost wordt in een iets groter volume en er een hogere druk ontstaat wordt er dan meer teststof in de hersenen gevonden? In het 2e experiment wordt gekeken of het juist andersom beter is door er eerst juist wat hersenvocht uit te halen, zodat ruimte ontstaat en dan de teststof in te spuiten. Deze studie, zal losstaan van maar, uitgevoerd worden voorafgaand aan een studie op gezonde vrijwilligers.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Aan het einde van dit project is vastgesteld met welke injectietechniek de hoogste concentratie van de teststof in het brein bereikt is. Hoe hoger de concentratie van de teststof in het brein hoe groter de kans dat de patiënt baat bij de behandeling heeft. Met als gevolg dat de symptomen van AS af zullen nemen of zelfs helemaal verdwijnen.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Voor dit onderzoek zullen 9 Java apen gebruikt worden omdat deze soort ook in voorgaande experimenten is gebruikt.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	De apen zullen ongerief ervaren door de injectie van de teststof. Echter neurale symptomen daarvan worden niet verwacht omdat de dosis die hier gebruikt wordt minimaal 5x lager is dan eerder getest. Bij deze test werden alleen voorbijgaande symptomen waargenomen. Daarnaast zullen ze stress ervaren van het transport voor het maken van de MRI op een externe locatie en door de narcose en het herstel hiervan voor de handelingen (PET-CTs, bloedafname en afname van hersenvocht).
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Het verwachte ongerief voor de dieren is matig en wordt voornamelijk veroorzaakt door transport en het aantal keer dat ze onder narcose worden gebracht.
3.6	Wat is de bestemming van de dieren na afloop?	Na afloop van het onderzoek kunnen de dieren in leven blijven en mogelijk hergebruikt worden voor andere onderzoeksdoeleinden.

4 Drie V's

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Het doel is het vinden van de optimale injectietechniek van een teststof voor AS welke een zo hoog mogelijke concentratie van deze teststof in de hersenen oplevert. Om de translatie van deze techniek naar de mens realistisch te maken is het noodzakelijk om een model te gebruiken dat zo dicht mogelijk bij de mens komt, qua anatomie en afmetingen. Java apen worden veelvuldig voor hersenonderzoek gebruikt vanwege hun grote overeenkomsten met de mens. Zodoende is het gebruik van de Java aap als model, in dit geval, niet te vervangen door knaagdieren.

4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Het aantal dieren zal zo laag mogelijk gehouden worden door de opzet van de studie zo optimaal mogelijk te maken. Dit laatste is mogelijk door data die verkregen is uit voorgaand onderzoek.
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	Onderzoek naar de werkzaamheid of verdeling van teststoffen in het brein kan in meerdere diersoorten gedaan worden. Echter vanwege de complexe interacties, is het brein van de Java aap bij uitstek geschikt omdat deze goed te vergelijken is met die van de mens. Middelen die in aanmerking komen om op apen getest te worden zijn vaak in de laatste fase van een ontwikkelingstraject en daardoor zeer specifiek voor de mens, waardoor ze minder goed of zelfs helemaal niet (meer) in knaagdieren werken. Daarnaast kan de verdeling van de teststof veranderen onder invloed van de hartslag, de ademfrequentie en lichaamsbewegingen. Hiervoor is het dus van belang dat een diersoort wordt gebruikt waarbij dit vergelijkbaar is ten opzichte van de mens.
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	De Java apen worden sociaal gehuisvest met voldoende kooiverrijking. Verbeteringen in de procedures en handelingen om het welzijn van de dieren zo hoog mogelijk te houden en de hoeveelheid ongerief te verminderen zijn meegenomen. Narcose wordt gebruikt wanneer dit noodzakelijk is en ditzelfde geldt voor pijnstilling. Daarnaast zullen de Java apen geëuthanaseerd worden wanneer ze (onverwacht) een humaan eindpunt bereiken voor het einde van de studie.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	