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10.2.e en 10.2.g

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 objective of this project is to understand how the cerebellum controls the covert attention shifts in cognitive tasks. We will focus on: (1) how the cerebellum controls covert attention shifts at the level of single neuron activity and (2) how the cerebellum is anatomically connected to the dorsal attention network.

The main aim of this application is to understand how the cerebellum controls covert attention shifts. To reach our aim, we need animals trained on a task in which they will have to make covert attention shifts. Both these sub-aims will be addressed in experiments with macaque monkeys. Experimental procedures required to reach both sub-aims are described in a single animal procedure 3.4.4.1.

Sub-aim 1: To investigate how the cerebellum controls covert attention shifts and through what (single neuron) coding strategies.

The control of attention shifts by the cerebellum on a single neuron level has never been investigated, although there is a vast amount of evidence from MRI, lesion studies and studies on cerebellar disorders that all indicate cerebellar involvement in this type of behavior. The high spatial temporal precision of micro-electrode recordings can teach us what computations individual cerebellar neurons make to execute trained behaviors. Switching off small parts of the network with reversible pharmacological interventions will show us whether a causal role of lobules VII and VIII exists in the control of attention shifts.

Sub-aim 2: To investigate the connectivity between the lateral cerebellum and attention controlling networks in the cerebral cortex.

A small number of anatomical studies have shown that connections between cerebral cortex and cerebellum are organized into discrete circuits or loops. For example, multiple cortical areas project to the cerebellum via the cortico-ponto-cerebellar pathway (Kelly and Strick, 2003). The cerebellum receives another big input from the cerebral cortex through the mesodiencephalic junction in the brainstem. DAN to cerebellar connectivity through this pathway has not been studied in primates. Using anatomical tracers, we will therefore investigate this pathway at the single neuron level. Furthermore, immunohistochemistry will be performed to determine which neurotransmitters are used by these cells to provide information on the excitatory or inhibitory character of the projecting neurons.

Feasibility of the projects

The experiments described in this protocol are based on techniques that have been established in our lab, both in rodents as well as primates. We have extensive experience in training animals on complex tasks, performing single neuron recordings and high-level data analysis and modelling. In addition, the experimental design is based on tasks that are known to provide robust behavioral outcomes (Ignashchenkova *et al.*, 2004). State-of-the-art equipment is present at the lab to perform the described measurements with the highest degree of accuracy. At the same institute, another research group is performing similar research on non-human primates for many years with great success. With them we share cutting edge animal facilities and operating rooms. As a close collaborator we often discuss the progress of our research, possible improvements to the tasks, caretaking and well-being of the animals.

3.3 Relevance

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

Scientific Relevance

The psychology of attention has been studied extensively (Pashler, 1999). The topic of representation and control of attention in the brain is one on which publications appear in the highest impact journals. The role of the cerebellum in the processes underlying attention, with some exceptions, have been neglected for many years. However, in recent years evidence has emerged on cerebellar involvement in many cognitive functions in health (Schmahmann, 2001; Moore *et al.*, 2017; Wagner *et al.*, 2017) and disease (Wang, Kloth and Badura, 2014). To formulate adequate hypotheses of how the cerebellum contributes to cognitive processes, it is essential to know the physiology and anatomy of these networks and how they are connected to other attention controlling regions in the brain. Since the first publication on covert attention in the cerebellum by means of fMRI (Allen, 1997), no attempts have been made to investigate how attention is controlled at a cellular level. These anatomical data could confirm the MRI based anatomical networks that have been discovered by means of rsMRI (Yeo et al., 2011). With this project, we will gain new fundamental insights into both the cellular processes in the cerebellum underlying control of attention, and their relationship to other networks. These results will be invaluable to acquire a systems level understanding of how attention is controlled by the brain as a whole.

Social relevance

Covert orienting attention is impaired in many groups of psychiatric patients including those suffering

from ADHD, autism, schizophrenia, dyslexia (Courchesne *et al.*, 1994; Maruff *et al.*, 1995). For instance, deficits in visual attention shifts have been shown to also impair reading performance (Casco, Tressoldi and Dellantonio, 1998). Furthermore, it has been suggested that impaired visual attention performance is one of the main factors underlying developmental dyslexia (Facoetti *et al.*, 2000; Bosse, Tainturier and Valdois, 2007). Patients with lesions to different parts of the cerebellum show a wide range of cognitive symptoms, including: impairment of executive functions such as planning, set-shifting, verbal fluency, abstract reasoning and working memory; difficulties with spatial cognition including: visual-spatial organization and memory; personality change with blunting of affect or disinhibited and inappropriate behavior; and language deficits including agrammatism (inability to construct a grammatical or intelligible sentence while retaining the ability to speak single words) and dysprosodia (variations in melody, intonation, pauses, stresses, intensity, vocal quality, and accents of speech) (Schmahmann and Sherman, 1998). This wide range of symptoms is commonly called cerebellar cognitive affective syndrome. The current study contributes to the understanding how the cerebellum performs some of these functions in a healthy state, which is as of yet a mystery. This knowledge may help gaining insight in how to treat some of these symptoms in diseased individuals.

3.4 Research strategy

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

General Research strategy

To explore how the cerebellum controls attention, we will perform neurophysiological recordings from individual cells (single unit recordings to detect spiking activity), transiently suppress the activity of neurons in parts of the network, and inject neuro-anatomical tracers. The combination of these techniques will provide better insight in how the cerebellar network that controls attention functions.

Sub-aim 1: To investigate how the cerebellum controls covert attention shifts and through what (single neuron) coding strategies.

Primates will be trained on a task in which they have to covertly shift their attention to a target in the peripheral visual field without making eye movements. Measurements on the activity of single neurons will provide us with a direct measure of the computations that cerebellum makes to achieve this behavior. Small variations in the task, such as variation in the amplitude of the stimulus, variable delays between task parameters or the addition of a correct or incorrect cue, will provide further insight in the dynamics of the network during the execution of the behavior. To establish a causal link between the cerebellum and covert attention shifts specific temporary blockers that are locally active will be administrated. The covert attention stimuli will be presented on both sides of the visual field, therefore when pharmacologically suppressing activity in one side of the cerebellum we will expect uni-lateral impairments of task performance where the contralateral side can function as control.

Sub-aim 2: To investigate the connectivity between the lateral cerebellum and attention controlling networks in the cerebral cortex.

We aim to elucidate the anatomical connections between the cerebral DAN (i.e. frontal eye field and intraparietal sulcus) and the cerebellar areas that are necessary for this task. After the neurophysiological experiments are finished, we will inject viral trans-synaptic neuroanatomical tracers in the cerebellar cortex. The advantage of viral tracers is that they can cross connected synapses. There are at least two synapses between the cerebellar cortex and the cerebral cortex. This poses a problem for non-viral tracers, as they cannot cross synapses and thus can only be used to elucidate part of the network. In contrast to MRI based anatomy, anatomical tracers provide the exact paths that individual neurons follow to communicated with each other. We will inject tracers that label cells which have connection towards and away from the cerebellum. The analysis will be carried out on brain tissue of the animals after they are euthanized and perfused with fixative. By looking at both routes, a networks level understanding will be obtained of the neural circuits controlling attention as a whole. This information could then be applied in interventions that treat some of the disorders mentioned in the social relevance

section.

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.

Basic habituation, chair training, and surgeries (AP 3.4.4.1)

A newly acquired animal follows a highly standardized training program, from habituating the monkey to the primate facility up to the point where they can perform visual tasks in daily training sessions. These initial stages have been carefully optimized and are performed or overseen by the well-trained technicians and animal caretakers of the facility. This standardization ensures a very high success rate acclimatizing monkeys to the facility and the training routine. Monkeys are obtained from the national primate center or, in exceptional cases, from licensed importers if there are no appropriate animals available at the primate center. They are socially housed in the facility, typically in pairs.

After habituation to the new environment, monkeys are trained to move into a primate chair that allows them to be transported comfortably from their home-cage to the experimental set-up. Once the animal can sit quietly in the chair for periods of >1 hour the animal undergoes a surgery to implant a head-post. The head-post is a small rod attached to the skull of the animal, it is used to fixate the head of the monkey during training and later electrophysiological recordings. This is an essential step because we train the monkeys to control their eye position and these measurements are only possible if the monkey's head is fixed.

After recovery from the head-post surgery, the animal is habituated to having his head fixated in the chair. Most monkeys adapt very quickly to this step. The monkey then starts daily training sessions in which it acquires juice rewards for performing simple eye-movement or hand-movement based tasks. Initially the tasks are very simple, such as directing his gaze ('fixating') on a large dot on a computer screen for a few hundred milliseconds. During this process, the animal will be placed on a controlled fluid uptake regime. Gradually the difficulty of the tasks is increased by making the dot smaller until the animal can fixate, then make guided eye-movements towards visual targets or hand-movements after the presentation of a 'go' cue. At this stage, the animal is ready to be trained on the experimental tasks specific to each sub-aim.

Neural recordings and inactivation during attention demanding task performance (AP 3.4.4.1)

The animals will be trained on a task where they have to shift their attention without moving their eyes. During the task, measurements on single cells will be made with acute micro-electrodes inserted for the duration of the recordings via the implanted recording chamber over the cerebellum. The cellular activity can be correlated with different task parameters to gain insight in what kind of computation are made by the cerebellum that are related to the attentional shifts. In other experiments, the performance on the task will be studied when activity in lobules VII and VIII of the cerebellum is suppressed through application of pharmacological agents, such as the selective GABA agonist muscimol. Dosage and volumes injected will be adopted from the literature, since many studies exist that apply these agents during task performance in the cerebellum. These agents, depending on the specific drug, work on the time scale from minutes to hours to a full day. This permits studying task performance before, during and after application, giving insight in whether the cerebellum is necessary to perform the task.

Neuro-anatomy of connectivity between the dorsal attention network and cerebellum (AP 3.4.4.1)

To elucidate the anatomical connections between the cortical DAN and the cerebellum, viral trans-synaptic tracers will be injected in the cerebellar cortex. These tracers can be injected through the recording chamber in the same way as the pharmacological agents. For the injection of viral tracers bio-safety level 3 facilities are necessary. Since these facilities are not present at the Institute, the animals will be transported by car to another Institute. After the injection, the animals will remain there to recover for a few days during which the tracer will spread through the nerve cells, after which the animals will be euthanized and transcardially perfused to fixate the tissue. Subsequent staining of tissue sections will give a precise picture of the neuronal connections between the DAN and the cerebellum.

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.

Two animals from other previously finished experiments can be re-used in this proposal. They have been used in the saccade/anti-saccade experiments described before, however in the current proposal these animals will be used for a different scientific question. In the saccade/anti-saccades experiments a similar experimental approach was used as proposed here, including single unit recordings from the cerebellum. For these experiments both animals have had recording chambers implanted, which are still in good shape and in the right spot for the experiments planned in the current proposal. The animals are already habituated to the facility and used to performing cognitively demanding visual tasks, therefore they can be rapidity employed in the new experiments. The cumulative discomfort of the procedures these animals have gone through is classified as moderate. Re-use eliminates the delays associated with the surgeries and habituation required when a new monkey is used. If one of these animals has to be withdrawn from the study, a new monkey will be purchased.

The first stage in the project is to train the animals on the new task. This will take 6 months. When sufficient task performance has been reached (consistent correct responses above chance level), neuronal recordings will start (24-30 months). After successful recordings and correlations between electrophysiological data and behavior have been established, inactivation experiments will be performed (6 months), however there is no specific reason for this order. Since the neuroanatomical tracer experiments are terminal they will be done last. The use of neuro-anatomical tracers requires the animals to be euthanized and their brains need to be extracted for sectioning and histology (2 months). The described experiments are likely to last less than the 5 years, the maximum duration of a CCD project. However, when the possibility of an animal dropping out (~ 12 months delay), or implants needing replacement (~ 6 months delay) are taken into consideration significant delays can be expected.

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	**Cerebellar measurements of neural activity and tracer injections** training – electrophysiological recordings – intracranial injections of pharmacological agents – tracer injections - perfusion
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'.	10.2.g
1.2	Provide the name of the licenced establishment.	10.2.g
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 Type of animal procedure: **Measurements of neural activity in cognitive tasks and tracer injections.**

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.

The procedures described in this appendix concern 1) measurements of neural activity in tasks investigating the deployment of attention in dual-task paradigms and memory paradigms and 2) injections of neuroanatomical tracers followed by ex vivo analysis of the connections

Monkeys will be trained to perform complex cognitive tasks in which they must attend visual stimuli while and keeping their eyes fixed on a different location on the screen. They will be trained to make behavioural choices using an eye-movements. The animals will receive implants (recording chambers), which will allow us to access the cerebellum with electrodes for recording neural activity while the animal performs the task. We will use tungsten electrodes to record the activity of individual neurons. We will use pharmacology to locally alter the neuronal activity.
The primary outcome parameters are:
i) The behaviour of the animal. The animal's accuracy and reaction time on the behavioural tasks will be recorded. We also investigate how behaviour depends on alterations of neuronal activity.
ii) Neural activity recorded from the electrodes. All electrode types described here can record single- and multi-unit activity and well as the local field potential. We will examine the link between neural activity recorded in the cerebellum and the behaviour of the monkey.
iii) Neuroanatomical connections revealed by the injected tracers.

Two animals from previous experiments performed by our lab can be reused in this proposal. They have been used in the saccade/anti-saccade experiments described in the project proposal. In those experiments a similar setup was used as proposed here, including single unit recordings from the cerebellum. The recording chamber implanted for these experiments is still in good shape, and in the right spot to reach lobules VII & VIII, which we will target in the experiments proposed in the current proposal. The animals also are already habituated to the facility and to performing cognitively demanding visual tasks.

If one of the animals has to be withdrawn from the study, a new monkey will be purchased, the animal will be acquired and acclimatized to the primate facility (left part of figure 1). They will undergo structural anatomical scans to guide the design of the surgical implants. The animals will be implanted with a head-post, which allows the head of the monkey to be fixed in the experimental set-up. The animals will be placed on a controlled fluid regime and trained on basic tasks such as fixating on small regions of a computer screen and making eye-movements to visual targets. After reaching high levels of performance on these basic tasks the animal will then be trained on more complex attention-demanding tasks. Once trained on these tasks the animals will be implanted with a recording chamber targeting the cerebellum. The recording chamber will be used with electrodes for neurophysiological recordings and gives access to the cerebellum for pharmacological experiments. After recovery from the implantation, we will then begin electrophysiological experiments in which we record neural activity from the electrodes while the monkey performs the task.

The flow of animals through this animal procedure is described in the figure below:

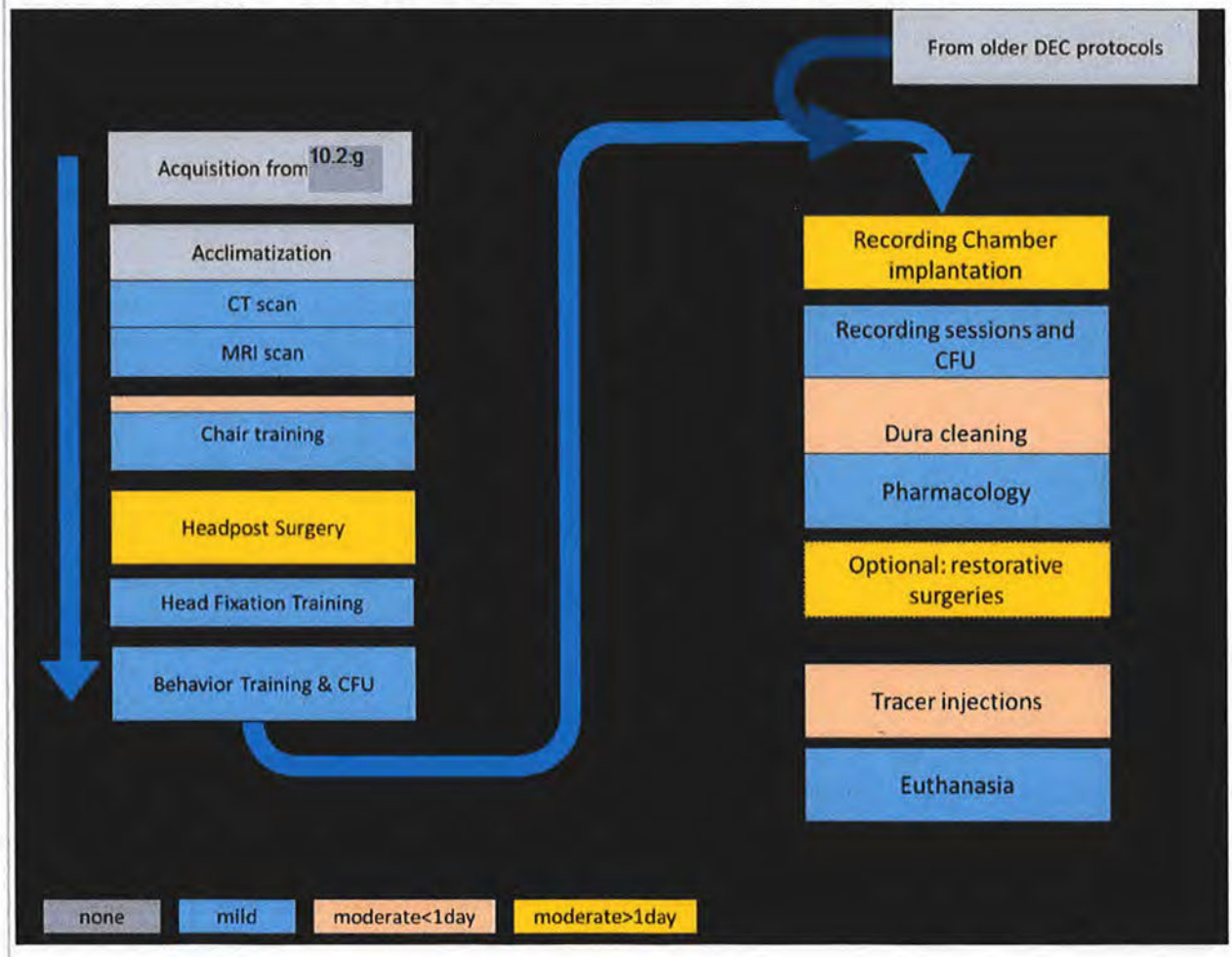


Figure 1 – A flow diagram outlining the steps of procedure 3.4.4.1. *The left part of the scheme is only applicable for a newly acquired monkey.* ***2 of our monkeys will be reused from old DEC protocols, for them only the right part is applicable.*** *CFU refers to periods in which the fluid uptake of the animal is controlled.*

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

The following procedures are described in the current appendix (3.4.4.1)

1. **Acquisition and housing**
2. **Acclimatization**
3. **CT scanning**
4. **MRI scanning**
5. **Chair training**
6. **First surgical procedure: Head-post implantation**
7. **Head-fixation training**
8. **Controlled fluid uptake**
9. **Behavioural training on basic tasks**
10. **Behavioural training on complex tasks**
11. **Second surgical procedure: Chamber implantation**
12. **Recordings sessions**
13. **Pharmacological Interventions**
14. **Removing tissue above the dura**
15. **Third surgical procedure: implant restorations**
16. **Annual health check**
17. **Transport and temporary housing at DM3 level**
18. **Injection of viral vector**
19. **Perfusion**

Note that step 1 thought 9 are only applicable if a monkey drops out and has to be replaced by a newly acquired monkey.

1. Acquisition and housing (only for a new animal)

A New monkey will be obtained from a licensed breeding facility. In all cases, we will first try to obtain animals from a national primate centre. Only under exceptional circumstances (no monkeys available at the primate centre) we will get them from a licensed importer. Monkeys will be housed in the primate facility of our institute. All animals are male and typically between 3-5 years of age when they arrive. This is the age at which young male monkeys typically leave their social group. We typically acquire two cage-mates together and these are then pair-housed for 3-4 weeks in a cage in isolation from the other monkeys (for quarantine reasons). When the results of viral and bacteriological tests are negative we can, if desired, pair these monkeys with established members of the group. We consult an ethologist from a national primate centre who advises us on appropriate pairings. This can be desirable to form stable pairings or larger groups (if the social character of the animals allows it). Our facility contains large cages and the monkeys will have access to a floor-to-ceiling play cage, which allows them to climb and swing. The play cage also contains a 'look-out' platform where the monkeys can view other monkeys in the facility. The environment will be enriched with toys (e.g. boxes filled with nuts or sweets, which the monkeys can fiddle out) and access to natural daylight. A TV screen shall be running in front of the cages during the day. A logbook will be maintained individually for each of the monkeys, carefully monitoring their general appearance, their eating behaviour, weight, and the performance during the training sessions.

2. Acclimatization (only for a new animal)

Discomfort: Mild or none

The monkeys will be adapted to the animal housing facility and the staff. This includes but is not limited

to an initial period in which the animal will be housed with a partner, will receive daily food treats from the staff, and will have access to toys in his cage and television. Previously acquired monkeys in the facility have successfully undergone this period of adaptation and interact well with the staff and do not exhibit signs of stress due to their environment. During this period, the monkey will receive a CT and MRI scan (see below). During one of these procedures the monkey will also be fitted with a collar, which is later used for guiding the monkey into a primate chair.

3. CT scanning (only for a new animal)

Discomfort: Mild (recovery from brief anaesthesia), once per animal (unless complications require re-assessment)

A CT scan may be obtained to allow 3D models of the monkey's skull to be constructed. These are used to custom design surgical implants which perfectly fit the skull of the animal. The monkey is anesthetized in its home cage, and then transferred to the CT scanner. The scanning procedure lasts less than 5 minutes. The monkey is then returned to his home-cage, and he is allowed to recover from anaesthesia. The total duration of the procedure is approximately 30 minutes. When there are no complications, this procedure will only take place once per animal. Occasionally, in the event that an implant comes loose, we may perform a further CT scan to assess the state of the underlying bone.

4. MRI scanning (only for a new animal)

Discomfort: Mild (recovery from brief anaesthesia), once per animal (unless complications require re-assessment)

Structural MRI scans are obtained to check whether the brain has any anatomical anomalies, and to localize specific brain structures and plan surgical implants. The monkey is anesthetized in its home cage and then transferred to the MRI facility. The anatomical scan lasts approximately 15-20 minutes, after which the monkey is returned to his home-cage, and allowed to recover. If there are no complications, this procedure will only need to be performed once per animal.

5. Chair training (only for a new animal)

Discomfort: Moderate the first 1-2 times, none after this.

The collar will be used to gently guide the monkey into the primate chair. Food and liquid rewards will be used in order to classically condition the monkey to enter the chair. Once learnt, the monkeys usually get into the primate chair voluntarily and rapidly. Once this behaviour is acquired, the animal will initially be rewarded with fruit or fruit juice for sitting quietly in the chair for short periods of time. The head of the monkey is not fixated at this stage. The time spent in the chair will gradually be increased as the animal becomes ever more comfortable and will be adjusted according to the animal's behavioural reaction.

6. First surgical procedure: Head-post implantation (only for a new animal)

Discomfort: Moderate for 1-2 days, becoming mild for 2-3 days.

All surgeries are performed in the purpose-built primate operation room within the primate facility of our institute. Specialist anaesthesia equipment is available and the surgeries are performed by trained staff. In order to head fix our monkeys during training, a head-post is attached to the skull. After induction of anaesthesia, an incision is made in the skin, and the skin is gently pulled aside, exposing the area of the skull above the cortex. The head-post is attached and the skin is sutured closed. Analgesics are given during the surgery. The duration of the procedure is approximately 1-2 hours.

At the end of the surgery, the animal is monitored and kept warm while waking up. Additional analgesics are given during the recovery period. Following the surgery, training will be discontinued for at least four weeks so that the animal may recuperate. After this time, the head-post is solidly fixed to the animal's skull.

7. Head-fixation training (only for a new animal)

Discomfort: Mild (but decreasing after 1-2 times), approximately 1 week

The animal will receive food and juice rewards for sitting quietly in the chair with their head fixated via the implanted head-post. The amount of time spent fixated in the chair will increase progressively and

will be modulated according to the behavioural reaction of the individual animal. Once the animal quietly sits in the chair with his head fixed for a sufficient period of time (0.5 hours), the animal will begin training on the basic experimental tasks. This step usually takes about a week, with the discomfort of the procedure being mild for the first one or two times that the animal is fixated, and lower after this.

8. Controlled fluid uptake (all animals)

Discomfort: Mild

To motivate the animals to work their access to fluid is controlled. The main reason why we use controlled fluid uptake is that we need to obtain a sufficiently large number of trials per session, for two reasons. First, we need reliable measures of the animals' perception which demands a large number of trials. Second, we obtain a larger number of trials to study the activity of neurons. The activity of neurons is inherently stochastic, i.e. the responses of a cell to repetitions of the same stimulus are variable, a stochasticity that is inherent to proper brain function. Controlled fluid access is by the far the most common method to motivate animals to perform cognitive tasks. We note that only healthy and cooperative monkeys that are at ease will perform these tasks in which they make eye or arm movements. Alternative methods have been explored as described by a workgroup for the British NC3R centre (National Center for Replacement, Refinement and Reduction of Animals in Research) (Prescott et al., 2010). These alternatives comprise 1) positive reinforcement with fruit juice, without controlled fluid uptake; 2) food-based reinforcement; 3) electrical stimulation of reward centres in the brain. Reinforcement with juice in the absence of controlled fluid uptake works well in the early stages of training when training sessions are short and tasks simple, but it is insufficient to motivate the animal to perform more difficult tasks or a larger number of trials. Food reinforcement with treats like raisins or peanuts is used in our lab to reward an animal for compliant behaviour (for instance, for coming to the correct compartment of the cage to interact with a researcher). It can also be used as reinforcement for short and simple tasks at the start of training. However, animals satiate quickly when rewarded with food and chewing movements cause artefacts in our recordings, which makes this type of reinforcement unsuitable for sessions that require many trials and precise recording of neural activity. Electrical stimulation of the reward centres involves an extra surgery with the accompanying risks of complications and direct electrical stimulation of the basal ganglia may interact with the neural processes that are the main focus of this application.

Controlled fluid uptake is thus the only viable method available to obtain sufficient numbers of trials to be able to reliably measure behaviour and neural activity. We implement controlled fluid uptake in a gradual fashion that adapts the level of fluid control to the behaviour of each individual. We begin with positive reinforcement using fruit juice without any controlled fluid uptake. We only use fluid control regimes if the animal is not sufficiently motivated to perform the task with no fluid control. We gradually introduce the fluid control with the aim to have the animals drink as much fluid as possible and the fluid control is only made stronger if necessary. Nevertheless, in the majority of animals it is necessary to restrict access to fluid to some level to obtain enough trials on the complex behavioural tasks described in the application. The amount of fluid control is individually determined for each monkey and we always begin by training animals without any fluid control. Most animals require some level of restricted access to fluid to motivate to perform behavioural tasks, and almost all animals require restricted access to motivate them to work on complex tasks. Our aim is to allow the animal to drink fluid during performance on the behavioural task until they are satiated. This is achieved by:

- The difficulty of the task is adjusted on each day so that the animal is able to receive fluid at a high rate, motivating him to work for more trials, and drink more fluid in total.
- The rate of fluid delivery is slowly increased during a training session to ensure that the animals drinks throughout the session.
- If the training session has to be aborted, for example due to a technical fault, then the animal receives fluid equivalent to the average intake during a training session.
- We investigate the preferences of each animal for particular rewards e.g. apple juice, different types

of fruit syrup, or water, and use a reward that is appealing to the animal

We take a number of measures to prevent dehydration:

- The monkeys always receive a minimum of 100ml of fluid each day. If this amount is not reached during the training session, it is supplemented.

- Averaged over a three-day period, the animals must receive a minimum of 35 ml per kilogram metabolic weight per day, this number is based on recommendations by the British N3CR (National Center for the Replacement, Refinement, and Reduction of Animals in Research) (Prescott et al., 2010) and the primate facility of UC Davis (2001). For example, a 10kg animal with a metabolic weight of $10^{0.75}$ kg must receive a minimum of: $10^{0.75}$ x 35 = 197ml of fluid per day, averaged over the previous three days. If this average is not achieved, the animal is supplemented with fluid. This is a minimum amount and the animals typically receive much more fluid than this.

- Fluid intake, both received during training and supplemented in the cage, is logged in an electronic system accessible by researchers, caretakers and inspectors.

- The animal is provided with fruit after the training session, the liquid content of the fruit is not counted towards the minimum amount.

- During breaks in the training schedule of more than one day (e.g. weekends) the monkey receives a full water bottle of at least 700ml, animals over 15kg receive an extra bottle. If the break is only one day, then the animal receives an amount of fluid equal to what it would typically receive during a training session.

- While the animal is under fluid control, the researchers and animal caretakers monitor its appearance and behaviour carefully every day, with checks by the animal caretakers during the weekend. We weigh the monkey before and after training and compare the weight to the average weight during the last week. The weight is also checked over longer intervals to prevent a slow loss of weight. We check the monkey for any signs of dehydration such as reduced skin tension, sunken eyes, either increased or reduced activity, and dry faeces. If any of these welfare criteria is abnormal, the monkey is taken out of training and provided with ad libitum access to fluid until it has recovered. In that case, the Animal Welfare Body will be informed so that they can check the animal. These criteria (weight, fluid consumed per day) are logged in an electronic system for each monkey so that the history is accessible.

- The animal receives a non-working period once every 9 weeks (on average over a year). During this period the animal is not trained and receives a full bottle each day (>700ml).

The British NC3R centre investigated in 2010 the use of controlled fluid regimes in brain research with macaque monkeys (Prescott et al., 2010). Their conclusion was that, when a controlled-fluid protocol is carefully applied and monitored, there are no negative consequences for the health of the animal. Follow-up research from the University of Newcastle (Gray et al., 2016) showed that controlled fluid uptake for 7 days per week did not lead to abnormal blood values or signs of dehydration. Another study (Hage et al., 2014) analysed a broad range of behaviours over several months during fluid control and found no evidence for alterations in behaviour, which indicates that the animals' wellbeing can be stably ensured during training sessions with a proper protocol. Indeed, from their general appearance, it is very difficult, if not impossible, to distinguish between monkeys under fluid control and monkeys with ad libitum access to water. Furthermore, the animals are seen regularly by a veterinarian to inspect their general condition, and we investigate measures of kidney function during the yearly check-ups. We have never obtained indications of impaired kidney function. Hence, our own experience is in accordance with the literature, which indicates that a careful protocol of controlled fluid uptake is a safe and effective manner to motivate animals to perform the required cognitive tasks.

Gray et al., 2016. Physiological, Behavioral, and Scientific Impact of Different Fluid Control Protocols in the Rhesus Macaque (Macaca mulatta). eNeuro 3(4).

Hage, S.R., Ott, T., Eiselt, A.-K., Jacob, S.N., Nieder, A., 2014. Ethograms indicate stable well-being during prolonged training phases in rhesus monkeys used in neurophysiological research. Lab. Anim. 48, 82–87.

Prescott M.J., Brown V.J., Flecknell P.A., Gaffan D, Garrod K, et al., 2010. Refinement of the use of food and fluid control as motivational tools for macaques used in behavioural neuroscience research: Report of a Working Group of the NC3Rs. J. Neurosci. Methods 193, 167–88

University of California Davis, 2001. Policy statement: water restriction in rhesus behaviour studies. UC Davis Office of Environmental Health and Safety.

9. *Behavioural training on basic tasks (only for a new animal)*
Discomfort: Mild

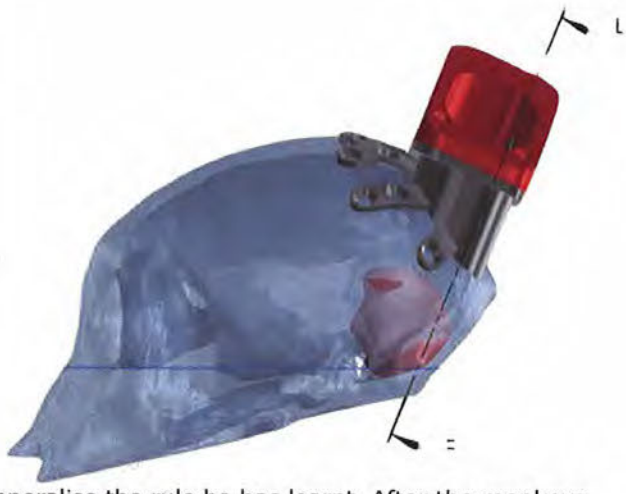
To motivate monkeys to perform their task, they are placed on a fluid control regime (described below). During training, the monkey is presented with sensory stimuli and responds with an eye movement and/or hand movement. We use positive reinforcement to train the animals, correct responses are followed by a fluid reward and the animals are allowed to work until satiated. The size of the reward is individually determined and is adapted throughout the training session to ensure that the monkeys remain motivated to work. No negative reinforcement is used, incorrect trials are typically followed by a lack of reward, and in some cases a small 'timeout' (5-10 s) may be given. As the monkeys learn the paradigm and their performance increases, we gradually make the task more challenging. Task difficulty is adjusted to ensure that the monkeys are able to obtain their full fluid ration during the training session. During the training periods, animals are typically in the setup 5 days per week, 1-4 h per day (typical is 2-3 h). Training on the initial tasks takes between 2-6 months depending on the monkey. An example of a training task is to have the monkey direct their gaze to a very small region of a computer screen for 1 s (known as 'fixation'). The difficulty of the task slowly increased by gradually decreasing the size of the area that the animal must fixate upon while slowly increasing the duration of the fixation. At the end of the training period the animal is expected to be able to fixate in a 1^0 diameter window for at least 400 ms and perform delayed saccade-tasks. A saccade is a very rapid eye-movement that monkeys and humans make approximately 3 times per second to direct the eyes to objects of interest. In a delayed-saccade task the animal must wait for a 'go' cue (e.g. the fixation dot changes colour) before making his eye movement. Most animals are also trained to make hand-movements in response to particular visual stimuli during the training period. The duration of this training period varies from 1 to 6 months depending on the aptitude of the monkey. In our experience, all monkeys are able to learn these tasks within 6 months.

10. *Behavioural training on complex tasks (all animals)*
Discomfort: Mild

The goal of the animal experiments described here is to understand how the deployment of attention is controlled by the cerebellum. To this end, we train the monkeys on tasks in which they have to attend to a particular visually presented object and report an aspect of the stimulus. Examples of such tasks include:

Attention tasks:

The animals will be trained to covertly shift their attention to a target and subsequently show that they have observed the target by making a saccade to a certain direction to obtain a reward. First, the animal has to fixate at a point in the centre of the screen. Then, a target appears in the periphery of the visual field. During the presentation of the target, the animal has to keep fixation on the central point and covertly shift its attention to the target to determine the direction to which he has to make a saccade to obtain the reward. Then there is a variable delay before the final saccade target is presented. The variable delay facilitates the decoupling of cellular activity related to the attention stimulus and the subsequent saccade. In the experiment, the amplitude of the attention shift is varied by changing the distance between the target and the fixation point. This permits decoding of the amplitude of the attention shift on the basis of the neural responses.
The difficulty of the task is slowly increased over days and the presentation of the stimulus is varied along several dimensions (e.g. size and position of the stimulus) to ensure that the monkey can



generalise the rule he has learnt. After the monkeys have reached high levels of performance on the complex tasks they will undergo operations to implant a recording chamber.

Figure 2. Chamber and head-post. *The image to the left shows a 3D reconstruction of the skull (viewed from the right) from a CT scan of a monkey who had been implanted with a head-post several months prior to the CT. The head-post had become very well integrated into the skull. These 3D models are also used to design recording chamber. The second image shows a recoding chamber over the cerebellum with in red angular adapter so the lateral parts of the cerebellum can be reached on both sides. The black line represents the path of an electrode reaching the cerebellum (red).*

11. Second surgical procedure: Recording chamber implantation and craniotomy (only new animal)

Discomfort: Moderate for 2-3 days, becoming mild for 1 week.

A surgical procedure is carried out under general anaesthesia to perform a craniotomy allowing access to the underlying brain structures. An incision is made in the scalp and the skin is retracted. A small section of skull (~2cm) is removed. A recording chamber is placed around the craniotomy and attached to the skull, with surgical screws and/or dental cement. Whenever possible the recording chamber will be 3D printed to ensure an excellent fit to the skull and will be made of titanium ensuring good bio-compatibility, strength and low weight. The animal receives appropriate analgesics during and after the procedure. The animal also receives drugs which reduce intracranial pressure during the operation (e.g. mannitol, dexamethasone). The duration of the procedure is approximately 1-2 hours.

The recording chamber must be cleaned every 2-3 days to prevent infection. The procedure takes place while the animal is sitting in the primate chair before or after a recording session. The chamber lid is removed and the interior of the chamber is flushed with anti-bacterial solutions such as chlorhexidine. Finally, the chamber is flushed with saline. The total duration of the cleaning procedure is around 5 minutes and it causes no discomfort.

12. Recording sessions (all animals)

Discomfort: Mild

The neural recording sessions follow an identical format to a behavioural training session, with the exception that the monkey is connected to the recording equipment. The electrode will be carefully moved across the dura and into the brain at the start of each recording session, this causes a brief

moment of mild discomfort. Subsequently, the electrode is moved into the tissue in micro-meter increments until the activity of a single cell is found. If pharmacological agents are used these will be applied via a combined recording-electrode/pipette as described I the next section. The animals will perform the same tasks as outlined above. The duration of daily sessions (max. 5 times per week) will be between 3-4 hours, including preparations. The total duration of the recording sessions will be between 2 and 2.5 years, although the chamber remains useable after this time-period.

13. Pharmacological interventions (all animals)

Discomfort: Mild

Reversible pharmacological agents will be applied locally through the implanted recording chamber, to the area of the cerebellum where task related neurons are recorded. A sharp glass pipette or combined electrode/pipette will be lowered across the dura in the same manner as a recording electrode. The pharmacological agent will either be slowly injected using pressure, or applied using iontophoresis. The approach used will depend upon the properties of the drug to be applied and the desired volume of the effect, iontophoresis produces a more local effect whereas pressure injections can affect larger volumes. For pressure injections the pipette will be connected to a small-volume syringe and a small quantity (<100nL) of drug will be slowly injected over the course of 5-10 minutes. For iontophoresis a small wire will be introduced into the glass barrel of the pipette. A holding current is applied to the wire to retain the drug within the barrel. To eject the drug, the current is switched in polarity and the charged particles of the drug are driven into the neural tissue. We will monitor the effect of the drug on neural activity through a recording electrode in an identical manner to that described above. Depending on the type of drug, suppression of activity can be up to 24 hours, therefor no more than 3 sessions per week can be performed to ensure complete washout before starting a session.

14. Removing tissue above the dura (all animals)

Discomfort: Moderate for 1 day (recovery from anaesthesia)

It is periodically necessary to remove tissue that has grown over the dura within a recording chamber to improve the ease with which electrodes can be moved into the brain. The monkey is lightly anesthetized and the tissue is removed with a specially designed tool. The amount of tissue damage caused by this procedure is minimal and the monkey recovers within an hour after cessation of the anaesthesia. The monkey receives analgesics during and after the procedure. The frequency of occurrence is approximately once per 3-6 months.

15. Third surgical procedure: Restorative surgeries (all animals)

Discomfort: Moderate for 1-2 days, becoming mild for 3-4 days.

In rare cases an implant (i.e. head-post or recording chamber) may become loose. A repair surgery is then performed to prevent failure of the implant. The repair surgery is performed under general anaesthesia with appropriate analgesia. The nature and duration of the repair surgery depends upon the type of implant and the extent of the problem, but the tissue damage imposed by the procedure is typically minimal. An individual monkey can undergo a maximum of two restorative surgeries per implant (including the head-post) during the course of these procedures. Repair surgery will always be performed in consultation with the Animal Welfare Body (IvD) and (if necessary) the veterinarian.

16. Annual health-check (all animals)

Discomfort: Mild (recovery from brief anaesthesia).

Once per year, each animal in our facility is checked by the veterinarian to assess their general health and appearance and to take blood/urine samples for further testing. In this way, the long-term health of the animals is closely monitored. The animal is lightly anesthetized during this procedure which takes 10-15 minutes per animal.

17. Transport and temporary housing at DM3 level (all animals)

Discomfort: Mild

During injections of viral vectors, it is necessary to move the monkey to a DM3 biological safety level facility due to GGO legislation. To this aim the animals will be transported to a primate DM3 facility in the Netherlands. Animals will always be transported and housed together with their cage-mate (who will also be assigned to this license) to reduce social stress. The animals will first be trained to sit quietly in the specialized transportation cage for periods of 1-2 hours by associating the transport box with positive rewards such as fruit. They will consequently not be anesthetized during the transportation and experience only mild discomfort.

18. **Injection of viral vector (all animals)**

Discomfort: Moderate for 1 day (recovery from anaesthesia)

During injections of viral vectors, it is necessary to move the monkey to a DM3 biological safety level facility. To this aim the animals will be transported to a primate DM3 facility in the Netherlands. To inject the vector, the monkey will be anesthetized. Injections will be made through the recording chamber, using an injection needle. A small gauge needle will be connected to a small-volume syringe and a small quantity (few microliters) of neuro-anatomical tracer will be slowly injected over the course of 5-10 minutes. Subsequently, the pipet will be slowly retracted to prevent spreading of the tracer to the non-target area. Since the injections are made through the recording chamber, no extra surgery is necessary. If larger volumes of vector are required, then 'convection enhanced delivery' techniques will be used: A thin cannula will be inserted into the brain and an infusion pump will be used to slowly deliver the viral vector into the brain. The animals will be housed in the DM3 facility until the viral trace has spread through the neurons, after which the animals will be euthanized and perfused.

19. Perfusion (all animals)

Discomfort: Mild or none

Due to the neuro-anatomical tracer injections animals will have to be euthanized and perfused at the end of the experiments. The animals are euthanized by an overdose of barbiturates and are then transcardially perfused with fixative.

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

The statistics in our studies are performed across neurons and behavioural trials, and we perform multiple recording sessions per animal meaning we can record a sufficient number of neurons with only two animals. Two is the absolute minimum number of animals that can be used to check for consistency across animals and is accepted as the norm in primate research. Although we have good experience with most of our monkeys, some individual circumstances may preclude a monkey from being used for a specific experiment (e.g. if he is not able to learn the behavioural task), or ambiguous results may require measurements in a third animal. In such cases, we will apply to the IvD of the institute for permission to use a third monkey.

B. The animals

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

Species used:

We will use rhesus macaque monkeys (*Macaca mulatta*) in these experiments. All monkeys are obtained from a national primate centre, or in exceptional circumstances (i.e. if no animals are available from a primate centre) from a licensed importer. Monkeys are typically acquired aged 3 years or older. The main aim of this application is to understand how visuospatial attention is controlled by the cerebellum and the connections to the cortical attention related areas. This approach requires a species with comparable neuroanatomy to the human and the ability to perform attentional tasks. Rodents are able to perform some simple cognitive tasks, however there are critical differences between the functioning of the rodent

and primate visual system which makes rodents unsuitable for this study. Primates have a region of their retina with extremely high spatial resolution known as the fovea, which is used for all detailed daytime vision. Primates constantly (3 times per second) make saccadic eye-movements so that the fovea of each eye are pointed at interesting regions of the visual scene. The mechanisms of visual attention are closely related to the mechanisms of eye-movement control and attention can be viewed as a 'pre-selection' of an object to plan an upcoming eye-movement. Importantly primates are able to withhold the actual eye-movement allowing them to covertly attend a particular object while keeping their eyes fixated on a different object. In rodents the control of the eyes is very different. The rodent retina is broadly speaking uniformly sensitive; they have no fovea. This means there is no need to move the eyes to fixate particular regions in the visual scene. Rodents very rarely make eye-movements and if they do these appear to be related to changes in arousal state. It appears therefore that rodents lack a mechanism for visuospatial attention making it impossible to study this process in these species. Cats have also often been used in basic visual studies, but these animals cannot be trained to perform covert attentional tasks. The experiments are invasive as they require the implantation of a head-post and further surgical implantations to allow stimulation electrodes to enter the brain. These experiments can therefore not be performed in humans. Macaque monkeys show very similar performance to humans on visual attention tasks and there is a large amount of literature on attentional processing in this species. We already have a broad outline of the anatomy of the attentional control system in macaques meaning we will be able to relate our results to previous findings making interpretation of the results much more powerful. Given these considerations, no alternative to the macaque monkey is available.

Sex used:
We exclusively use male monkeys in these studies. Our facility houses only male monkeys as males adapt better to living in paired social housing than females and there are no possible complications with breeding that would be present with female animals. The choice for males will not affect the results of the study as it is highly unlikely that there are differences between the sexes in how attention is controlled. Males are therefore chosen to allow us to maintain 100% male animals in our facility.

Animal number:
We expect that at least two animals are necessary in order to obtain reliable results for each experimental question considered. When comparable data is obtained from two individuals it can be assumed that the results are not attributable to individual differences.

The experiments described here will be used to address two sub-aims:
1) How does the cerebellum control covert attention shifts?
2) How is the lateral cerebellum connected to attention controlling areas in the cerebral cortex?

Given that to answer the second research question we can use the animals from the first after that research question is completed we will use two animals in the course of this proposal. These two animals are now present under a protocol from the old laws, but will be moved to this protocol once it has been approved.

Previous studies have obtained reliable results from two animals per question, but given the novelty of the proposed experiments it remains hard to estimate the individual variability that we will encounter. It could be possible that data from one animal must eventually be excluded from the analysis, contradictory results arise from the first two monkeys, or ethical considerations require the termination of one animal before conclusive data is gathered. Such cases require the acquisition of data from a third animal. The acquisition of a third animal for a particular research question will be performed in consultation with the IvD of the institute. Another group examined the use of monkeys in their lab over the past 10 years and found that in 4 out of 18 projects a third monkey was required (22% chance). Given this value it is likely that we will require one extra animal in addition to the two animals above. We therefore require a potential maximum of 1 newly acquired animal making a total of 3 animals.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

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

Two animals from other previously finished experiments can be used in this proposal. They have been used in the saccade/anti-saccade experiments described in the main proposal, however in the current proposal these animals will be used for a different scientific question. In the saccade/anti-saccades experiments a similar experimental approach was used as proposed here, including single unit recordings from the cerebellum. For these experiments both animals have had recording chambers implanted, which are still in good shape and in the right spot for the experiments planned in the current proposal. The animals are already habituated to the facility and used to performing cognitively demanding visual tasks; therefore they can be rapidly employed in the proposed experiments. The cumulative discomfort of the procedures these animals have gone through is moderate.

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

☒ No

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

D. Replacement, reduction, refinement

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

Replacement

The main aim of this application is to understand how the brain controls the deployment of attention in cognitive tasks. Many studies have approached this question by using cognitive neuroscientific techniques in humans. These studies have provided valuable information about the brain areas which are involved in allocating attention and have led to a number of proposed models about the neural mechanisms of attentional deployment. Unfortunately, the temporal resolution of fMRI is not sufficient to track activity in cognitive tasks and the spatial resolution of EEG/MEG is not sufficient to localize the neural activity to particular brain regions. To fully understand the neural mechanisms that engage and shift attention we need a technique with both high temporal and spatial resolution such as invasive electrophysiological recordings. Invasive recordings can only rarely be performed in humans, typically in surgical investigations of epileptic patients who are implanted with depth electrodes to localize the source of the epileptic activity. In these studies, the location of the electrodes is based purely on clinical criteria, and they are very rarely placed in areas involved in attentional control such as the FEF or parietal cortex or cerebellum. This makes it impossible to collect sufficient neural data to gain an understanding of the underlying neural mechanisms. It is therefore not possible to replace the experiments described here with experiments in humans. It is also impossible to replace the monkeys in these experiments with rodent models or cats for the reasons outlined above in section B. Briefly, rodents and cats are unable to perform the covert attention tasks, which are essential to understanding the mechanisms of attentional deployment. Given these considerations, no alternative to the macaque monkey is available.

Reduction

The number of animals we want to use is the minimum number with which reliable results can be obtained, and no further reduction is possible. If possible, we can implant record in the other hemisphere after recordings are no longer possible from the original sites due to damage of the tissue from multiple penetrations. This allows us to increase the total number of recordings made and behavioural data collected from one animal. Similarly, we will reuse animals from an older license for the studies described here. We hope to be able to complete the study with only re-used animals, thus we require maximally 1 new animal to answer our research questions, and expect that 2 will come from other experiments. See the flow-chart in Figure 1 for further details. Several of the techniques described below under 'refinement' also contribute to a reduction in animal numbers as they improve the stability of implants allowing sampling of more data so that, if possible, the research question can be answered using data

from only two animals.

Refinement

All procedures (including housing) are refined to minimise discomfort for the animals as much as possible, using the latest knowledge and techniques in animal welfare. We take several measures to refine our experiments. First, we decrease the amount of stress by gradually introducing all aspects of the behavioural tasks, and careful conditioning of the monkeys to any novel aspects of our behavioural experiments. Second, we implement an elaborate set of measures (described above) to prevent adverse effects of the controlled fluid uptake. Third, all surgeries will be carried out by persons who are well trained. Fourth, our implants (such as head posts and chambers) are custom-designed to the anatomy of individual animals, 3D printed, and coated with biocompatible material that promotes bone growth, enhancing the integration of implants with the skull. Fifth, animals are housed socially in an enriched environment in order to keep them engaged, reduce the discomfort of living in a cage, and improve their cognitive abilities.

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

During surgeries, analgesics and anaesthesia are used to minimize pain and suffering. Breathing and temperature will be registered and level of anaesthesia and warmth of heating-pad will be adjusted as such. Post-surgical analgesics will be administered and animals will be kept on a warm blanket or under a warm lamp until they wake up. Food and fluids are placed in the home cage to facilitate easy access to food and water. They will be allowed to recover for several weeks following surgery. Behaviour, wound area and appearance will be monitored daily for at least 7 days post-surgery.

After the recovery period, we constantly monitor the welfare of trained animals, assessing their appearance and behaviour every day.

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.

The proposed experiments are novel and an essential step to understanding the neural mechanisms that underlie the deployment of attention. The applicants are very familiar with the research literature on visual attention and the present set of experiments are ground breaking and have not been performed previously. The proposed experiments are fundamental research, and are not legally required.

Accommodation and care

F. Accommodation and care

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

☐ No

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

Monkeys will be housed solitarily for several days following an implantation surgery, to ensure full rest and recovery. The cage environment will be enriched by bedding material, swings, toys, and treats. We will monitor their weight and appearance on a daily basis.

G. Location where the animals procedures are performed

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

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

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

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

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

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

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

During surgery, anaesthesia and analgesia will be applied as described above. Also post-surgery analgesics will be administered.

I. Other aspects compromising the welfare of the animals

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

1. Infections: In rare cases there is a possibility of infection around the wound area. In these cases, we will apply additional analgesics and/or antibiotics. Visible signs of microbial infection will be monitored. The following will be considered as signs of an unhealthy state of the animal: (a) aberrant behaviour; (b) dehydration; (c) weight loss.

2. The occurrence of weight loss due to the controlled fluid uptake and the measures that we take to prevent dehydration have been described above.

3. Insufficient recovery after surgery: applicable if an animal shows permanent weight loss (more than 15%-20% of the weight immediately after surgery for more than 10 days). This occurs infrequently (<2%).

4. Loosening of an implant.

5. Brain swelling during operations.

6. Seizures. In very rare cases it may be possible an animal suffers from a seizure, this may be due to brain-swelling after an operation or a side-effect of an infection.

7. Sub-dural bleeding. During operations or electrode penetrations it can occur that a blood-vessel is damaged. Small bleeds typically cease within minutes with no ill effects. Very rarely the bleed may be larger leading to neurological symptoms. These typically disappear within a few days and produce no more than moderate discomfort. In the case of persistent neurological symptoms, or the possibility that the animal will experience more than moderate discomfort, the animal is euthanized.

Explain why these effects may emerge.

Surgical implantation of cranial and brain implants is accompanied by risk of microbial infection, tissue rejection, or unwanted growth of granulation tissue that prevents the implant from integrating with the body. The causes of the other adverse effects are described above.

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

1. We constantly monitor the animal's behaviour, liquid intake, wound area, and physiology. Surgeries are performed under sterile conditions and without any unnecessary delays to minimize the amount of time the animal spends under anaesthesia. Animals will be monitored daily and if adverse effects are present, this will be discussed with the veterinary officer. If necessary, treatment will be initiated

(topically or systemically applied medication).

2. The measures to prevent dehydration due to controlled fluid uptake have been described above.

3. We monitor animals carefully after surgery. They are placed under heat-lamps during recovery and given post-operative analgesics. Food and water are freely available in the home-cage. The animals are temporarily housed alone after the surgery to allow proper recovery. All animals are checked once per year by the veterinarian to monitor their long-term health.

4. Over the years we have made several refinements to our cranial implants, such as head posts (described above in the section on refinement) and we continuously review and refine the design of our implants. Head posts and all cranial implants are now custom-designed for each monkey and 3D printed to ensure a good fit. This greatly reduces the chances of the implant becoming loose. In the unlikely event that a head post or array connector becomes loose or detached, the animal is closely inspected, and may undergo a CT scan to allow us to assess the condition of the bone. If an implant becomes loose/detached from the skull, we reattach the implant in a repair surgery. The repair surgery is performed under anaesthesia and with analgesia in an identical fashion to the original attachment surgery. We estimate the discomfort to be moderate during recovery from the anaesthesia (1 day) becoming mild for 1-2 days. In rare cases (once in the past 10 years), the skull becomes infected, causing moderate discomfort. In these cases, the monkey is immediately euthanized under anaesthesia. We minimize the occurrence of head post failures by slowly adapting the monkey to being head-fixed in the set-up so that it is relaxed and does not exert strong forces on the headpost while fixed in the setup.

5. To combat the possibility of brain-swelling, we always give pre-, peri- and post-operative corticosteroids for operations in which the skull is opened and, if indicated, we give intravenous mannitol.

6. If the animal suffers a seizure, anti-seizure medication is given immediately and the underlying cause is treated, e.g. with high-dose corticosteroids or antibiotics. The veterinarian is informed.

7. All operations are performed as precisely as possible by trained staff.

J. Humane endpoints

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

☐ No > Continue with question K.

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

Each animal that undergoes surgery will be monitored for clinical parameters. The monkey will be monitored for their general appearance and activity level. If a monkey has an appearance that gives cause for concern (e.g. signs of infection around the wound, weight loss, reduction in activity level) then we will notify the Animal Welfare Body and evaluate the animal together with the veterinarian of the 10.2.g Similarly, if the animal does not recover well from anaesthesia we will evaluate the animal together with the veterinarian.

In addition, the weight of the animal will be monitored and if the animal loses 10-15% of their weight in 2 consecutive days then the veterinarian will be contacted and a decision will be made whether a humane endpoint has been reached.

If a cranial implant breaks off and infection or severe damage to the skull is sustained, the monkey will be immediately euthanized (this only occurred once in the past 10 years).

Indicate the likely incidence.

Based on previous experience, humane endpoints are expected to be met in 0-5% of the animals tested within the time frame of the experiments.

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').

Acclimatization (only new animals)

Discomfort: Mild or none

CT scanning (only new animals)

Discomfort: Mild (recovery from brief anaesthesia)

MRI scanning (only new animals)

Discomfort: Mild (recovery from brief anaesthesia)

Chair training (only new animals)

Discomfort: Moderate the first 1-2 times, none after this.

The animal experiences some stress the first one or two times, but they very quickly learn to associate the chair with rewards and voluntary enter the chair after this phase.

Surgery: Head-post implantation (only new animals)

Discomfort: Moderate for 1-2 days, becoming mild for 2-3 days.

Head-fixation training (only new animals)

Discomfort: Mild

Monkeys very quickly adapt to head-fixation.

Behavioural training (all animals)

Discomfort: Mild

Controlled fluid uptake (all animals)

Discomfort: Mild

These procedures are classified as mild, given that many measures are taken (described above) to ensure that the monkeys receive their daily fluid requirements.

Surgery: Recording chamber implantation and craniotomy (only new animals)

Discomfort: Moderate for 2-3 days, becoming mild for 1 week.

Recording sessions (all animals)

Discomfort: Mild

Pharmacological interventions (all animals)

Discomfort: Mild

Removing tissue above the dura (all animals)

Discomfort: Moderate for 1 day (recovery from anaesthesia)

Surgery: Restorative surgeries (if necessary)

Discomfort: Moderate for 1-2 days, becoming mild for 3-4 days.

Restorative surgeries cause considerably less discomfort than the original implantation surgery as there is no need to cut through the skin and muscle tissue, which have already been removed during the original implantation. Hence, the overall tissue damage is minimal and the animals recover rapidly. The surgery and recovery from surgery is classified as moderate discomfort (for 1-2 days), becoming mild (for 3-4 days).

Transport and temporary housing at DM3 level (all animals)

Discomfort: Mild

Surgery: Neuro-anatomical tracer injections (all animals)

Discomfort: Mild

Annual health check (all animals)

Discomfort: Mild (recovery from brief anaesthesia)

Perfusion (all animals)

Discomfort: Mild

Cumulative discomfort

The monkeys will undergo surgical procedures, which lead to discomfort at the lower end of moderate that is mostly associated with the recovery from anaesthesia and lasts a maximum period of 1-2 days. Previous research indicated that there is no indication that successive procedures cause cumulative suffering that is more than that caused by the individual procedures and that there is no increase in discomfort through incomplete recovery between events ('stacking') or potentiation of adverse effects and suffering by earlier procedures (Pickard, 2013). Many animals instead showed signs of diminished responses to repeated procedures such as restraint and handling.

Furthermore, we have taken several measures to exclude the possibility that the cumulative discomfort

can exceed the moderate level:
(1) The behaviour and health of the animal is carefully monitored by the researchers, the care-takers and experienced vets and entered in an electronic database, which includes the general appearance of the animal, its weight and the amount of food/drink. To exclude the longer-term ethological and psychological adverse effects on the animals' wellbeing (i.e. not to the animals' medical condition), we will ask the opinion of an expert monkey ethologist on a regular basis. If there is a threat that the future cumulative discomfort level of an individual animal might exceed moderate (for medical or psychological/ethologic reasons), the animal will be taken out of the experiment (rehomed or euthanized) thereby excluding the possibility of severe cumulative discomfort.
(2) Surgical procedures are followed by a minimum of four weeks of recovery. In practice, the interval between successive surgeries is a few months, ensuring that the monkeys have fully recovered after the surgeries before any further interventions take place.
(3) Social housing of a high standard. The animals live in stable social pairs in large floor-to-ceiling cages with natural daylight. The cages are enriched with toys and puzzles and the animals engage in their natural behaviours such as grooming, climbing and foraging for food (e.g. for peanuts hidden in the sawdust on the floor). We see no evidence for stereotypical movements or any evidence that long-term housing causes any suffering for the animals.
(4) Controlled fluid protocol approach. Our approach to controlled fluid uptake is to use the mildest form of fluid control necessary to achieve the desired performance of the animal. In the answer to the question above we outlined why the amount of discomfort associated with this procedure is maximally mild.
The (cumulative) discomfort level classification 'moderate' is also in accordance with (a) the opinion of our vets, who have ample experience with experiments in monkeys and rate the cumulative discomfort as moderate, (b) we inspected the retrospective assessments of the discomfort experienced by monkeys in similar experiments of the previous five years, and found that they have always been in the moderate category, (c) the opinion of the IvD and (d) our DEC protocols under the previous law have always been at the moderate discomfort level while using similar techniques. The discomfort level has actually become lower over the years, due to refinements of the implants, anaesthesia protocols, measurement techniques and enrichments in the monkeys' environment. Even though this is unlikely, if there is a threat that the future cumulative discomfort level of an individual animal might exceed moderate (for medical or psychological/ethologic reasons), the animal will be taken out the experiment, thereby excluding the possibility of severe cumulative.

These animals will be re-used from previous experiments, therefore they already possess the required implants. The discomfort they will undergo from the current experiments is classified as mild with a few short (<1 day) periods of moderate discomfort. Thus, the total discomfort that animals will face during their experimental life, with previous experiments included, is moderate. If the proposed experiments would be executed with new animals the total discomfort would be higher, since they would have to undergo the surgical procedures necessary to prepare them for the experiments.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

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

Histological analysis needs to be performed on the brain to analyse the neuronal pathways as integral part of the study. Therefore, animals will be euthanized at the end of the experiment. The animals are euthanized by an overdose of barbiturates and subsequently transcardially perfused with fixative.

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

Centrale Commissie Dierproeven

5

> Retouradres Postbus 20401 2500 EK Den Haag

10.2.g
10.2.e en 10.2.g
10.2.g

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
10.2.g 20184587
Bijlagen
2

Datum 17 januari 2018
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte 10.2.e en 10.2.g

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 16 januari 2018. Het gaat om uw project "Understanding control of covert attention by the cerebellum – 10.2.e en 10.2.g Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD10.2.g 20184587. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Datum:
17 januari 2018
Aanvraagnummer:
10.2.g 20184587

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
17 januari 2018
Aanvraagnummer:
10.2.g 20184587

Gegevens aanvrager

Uw gegevens

Deelnemersnummer NVWA:	10.2.g
Naam instelling of organisatie:	
Naam portefeuillehouder of diens gemachtigde:	10.2.e en 10.2.g
Postbus:	10.2.g
Postcode en plaats:	

Gegevens verantwoordelijke onderzoeker

Naam:	10.2.e en 10.2.g
Functie:	10.2.e en 10.2.g
Afdeling:	10.2.e en 10.2.g
Telefoonnummer:	
E-mailadres:	

Gegevens plaatsvervangende verantwoordelijke onderzoeker

Naam:	10.2.e en 10.2.g
Functie:	10.2.e en 10.2.g
Afdeling:	10.2.e en 10.2.g
Telefoonnummer:	
E-mailadres:	

Datum:
17 januari 2018
Aanvraagnummer:
10.2.g 20184587

Over uw aanvraag

Wat voor aanvraag doet u? [x] Nieuwe aanvraag
[ ] Wijziging op een (verleende) vergunning die negat
gevolgen kan hebben voor het dierenwelzijn
[ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum: 1 februari 2018

Geplande einddatum: 31 januari 2023

Titel project: Understanding control of covert attention by the cerebellum – 10.2.e en 10.2.g

Titel niet-technische samenvatting: De rol van de kleine hersenen in de aansturing van aandacht en de connectiviteit met de grote hersenen.

Naam DEC: 10.2.g

Postadres DEC:

E-mailadres DEC:

Betaalgegevens

De leges bedragen: € 1.285,-

De leges voldoet u: na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:
[x] Projectvoorstel
[x] Beschrijving Dierproeven
[x] Niet-technische samenvatting

Ondertekening

Naam: 10.2.e en 10.2.g

Functie:

Plaats: 10.2.g

Datum: 16 januari 2018

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

10.2.g
10.2.e en 10.2.g
10.2.g

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
10.2.g 20184587
Bijlagen
2

Datum 17 januari 2018
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 17 januari 2018
Vervaldatum: 16 februari 2018
Factuurnummer: 184587

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD10.2.g 20184587	€ 1.285,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL29INGB 070.500.1512 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

4587

6

Centrale Commissie Dierproeven

17 JAN. 2018

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☒ Ja > *Vul uw deelnemernummer in* 10.2.g

☐ Nee > *U kunt geen aanvraag doen*

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

Naam instelling of organisatie	10.2.g
Naam van de portefeuillehouder of diens gemachtigde	10.2.e en 10.2.g
KvK-nummer	10.2.g

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

Straat en huisnummer	10.2.g
Postbus	
Postcode en plaats	
IBAN	
Tenaamstelling van het rekeningnummer	

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	10.2.e en 10.2.g ☒ Dhr. ☐ Mw.
Functie	10.2.e en 10.2.g
Afdeling	10.2.e en 10.2.g
Telefoonnummer	
E-mailadres	

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	☒ Dhr. ☐ Mw.
Functie	10.2.e en 10.2.g
Afdeling	10.2.e en 10.2.g
Telefoonnummer	
E-mailadres	

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters Functie Afdeling Telefoonnummer E-mailadres	☐ Dhr. ☐ Mw.
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag* ☒ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3 ☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2 ☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier ☐ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☐ Nee > Ga verder met vraag 3 ☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum Einddatum	1 - 2 - 2018 31 - 1 - 2023
3.2	Wat is de titel van het project?	Understanding control of covert attention by the cerebellum.	
3.3	Wat is de titel van de niet-technische samenvatting?	De rol van het cerebellum in de aansturing van aandacht en de connectiviteit met de cerebrale schors. (X)	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC Postadres E-mailadres	10.2.g

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1285 Lege
☐ Wijziging € 568 Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

☐ Via een eenmalige incasso
☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☐

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam 10.2.e en 10.2.g
Functie
Plaats 10.2.g
Datum 11 - 1 - 2018
Handtekenir 10.2.e en 10.2.g

Format Versie 10 februari 2017
Datum: vrijdag 16 februari 2018

7

Format DEC-advies AVD [redacted] 2018 4587

Maak bij de toepassing van dit format gebruik van de Praktische Handreiking: Ethisch Toetsingskader voor proefdiergebruik. Voor voorbeelden, zie bijlage I.

Herhaling van antwoorden is niet nodig. Indien van toepassing kan verwezen worden naar een bij een eerdere vraag verstrekt antwoord.

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD[redacted] 2018 4587

2. Titel van het project: Understanding control of covert attention by the cerebellum.

3. Titel van de NTS: De rol van de kleine hersenen in de aansturing van aandacht en de verbindingen met de grote hersenen.

4. Type aanvraag:
   - ■ nieuwe aanvraag projectvergunning
   - □ wijziging van vergunning met nummer

5. Contactgegevens DEC:
   - naam DEC: [redacted]
   - telefoonnummer contactpersoon: [redacted]
   - e-mailadres contactpersoon: [redacted]

6. Adviestraject (data dd-mm-jjjj):
   - ■ ontvangen door DEC: 17-01-2018
   - ■ aanvraag compleet: 01-02-2018
   - ■ in vergadering besproken: 18-01-2018
   - ■ anderszins behandeld: niet van toepassing
   - ■ termijnonderbreking(en) van 22-01-2018 tot 01-02-2018
   - ■ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: niet van toepassing
   - ■ aanpassing aanvraag: versie met aanvullende informatie ontvangen op 01-02-2018
   - ■ advies aan CCD: 16-02-2018.

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

Bij de punten 8 t/m 10 kan worden volstaan met 'n.v.t.' wanneer de betreffende acties niet aan de orde zijn geweest. Bij vragen die gericht zijn op het compleet maken van de aanvraag (aanvullingen achtergrond informatie etc.) kan bij punten 8 en 9 worden volstaan met de vermelding van het type vragen en de vermelding dat de aanvraag op de desbetreffende onderdelen is aangepast of dat de antwoorden in de aanvraag zijn verwerkt. Bij vragen die gericht zijn op het verkrijgen van verklaringen voor keuzes die door de aanvrager gemaakt worden, kan niet worden volstaan met het weergeven van de strekking van de antwoorden tenzij de antwoorden volledig in de aanvraag zijn opgenomen. Als dat het geval is, moet dat in het DEC advies worden benoemd en in de aanvraag inzichtelijk worden gemaakt.

8. Eventueel horen van aanvrager:
   - Datum: 10.2.g
   - Plaats: [redacted]
   - Aantal aanwezige DEC-leden: 7
   - Aanwezige (namens) aanvrager: plaatsvervangende verantwoordelijke onderzoeker.
   - Gestelde vraag / vragen: De mondeling gestelde vragen zijn na afloop van de vergadering ook schriftelijk aan de aanvrager gestuurd als een verzoek om aanvullende informatie. Dit verzoek (zie onder punt 9; brief 22-01-2018) heeft geresulteerd in aanpassingen van de aanvraag.
   - Verstrekt(e) antwoord(en): zie punt vraag 9
   - Het horen van de aanvrager heeft wel/niet geleid tot aanpassing van de aanvraag wel; zie onder vraag 9.

9. Correspondentie met de aanvrager
   - Datum brief van de DEC: 22-01-2018.

Gestelde vragen (samenvatting) 22-01-2018:

Questions/remarks DEC

1. You plan to use of the monkeys that are already present at the Institute and it needs to be clear that the reason to use monkeys is in no way motivated by the fact that these two animals are available in the facility. The project will be evaluated as if two new monkeys are needed. You are of course allowed to point out the advantages of re-using these monkeys compared to buying two new ones.
   Reply: We have explained the relevance of this proposal and the fact that this submission is independent of the presence of above mentioned animals in the Institute (section 3.4.3 and Appendix 1).
   A statement elaborating on the advantages of using these particular animals has been provided (section 3.4.3).

2. The DEC would like to be informed about how the project ultimately contribute to an application in human given that the techniques used in monkeys cannot be directly applied?
   Reply: This issue has been addressed by adaptation of the section on social relevance, more information is provided on possible clinical applications. We also suggest some follow up experiments that could bridge the gap between monkey and human experiments upon completion of our project.

3. What is the hypothesis regarding the problems that people suffering from certain afflictions have with covert attention in relation to cerebellar defects? This information is needed to better understand/motivate the social relevance of the project.
   Reply: This issue has been addressed by adaptation of the section on social relevance, a strong link between cerebellum/attention/mental disorders in particular autism has been provided. In order to thoroughly address this question we have expanded the list of referenced articles and we now list study outcomes that corroborate this link.

4. How can it be determined that the monkey really shifted its attention without moving its eyes? What are the readout parameters? In your presentation a figure of the task was presented explaining this in a better way than the present Fig 1A. Also in the appendix; the explanation of the task used and the experimental design (correlations between task variations and changes in firing rates?) warrants more explanation.
   Reply: Section 10 of appendix (Behavioural training on complex tasks (all animals) has been extended on the topic of correlations between task variations and neuronal activity and the figure of the presentation has been added as figure 3.
   Read-outs of attention shifts have been added to research strategy/ sub-aim 1.

5. One behavioral task is described. Is this the only task or will more tasks be used or will there be subsequential iterations of this task?
   Reply: The described task will be the only task used for these experiments.

6. A point of concern is that in the end; n=2; is this sufficient to draw sound conclusions? How many cells per animal do you expect to need data from for the normal condition and in the context of pharmacological manipulation?
   Reply: Depending on the diversity and complexity of the neuronal responses between 30-100 cells per animal should be sufficient to draw solid conclusions. These numbers of cells are generally reported in the literature of comparable studies. N=2 is the standard number of animals for primate neuroscience research on a single task. This is enough to reach robust conclusions, since it is possible to record many more neurons over extended periods of time per animal in monkeys than in rodents.

7. Is comparable research ongoing elsewhere? This additional information should be added to Appendix 1 section E.
   Reply: We are collaborating with 10.2.e en 10.2.g at the technical level. He is also interested working on the eye movement control in NHP, but he is not focusing on the relation between cerebellar activity and attention as proposed in the proposed project.

8. The go/nogo between electrophysiological recordings and the final tracing studies is not clear? This additional information needs to be given under section 3.4.3. of the proposal.
   Reply: When robust results have been obtained in the electrophysiology and pharmacology experiments the neuro-anatomical tracer experiments will be done. Since the neuroanatomical tracer experiments are terminal they will be done last. If no effects have been found in the electrophysiology and pharmacology experiments, the anatomical experiments are still worthwhile considering that anatomical information on cerebro-cerebellar loops in primates is extremely rare and could be the foundation of much other research.

9. Provide additional information on why this research is of scientific interest. Which possible new research avenues could be opened?
   Reply: We expanded the scientific relevance section on the novelty value of the this proposal and stress that it will answer a question that has been open for more than 20 years.

10. What is the link between attention shifts and the mentioned disorders; which disorders are primarily relevant?
    Reply: The social relevance section's focus has been shifted to one cerebellar disorder: autism. This section now clearly shows the link between attention shifts – the cerebellum – autism.

11. Provide additional information on the go-no go decision regarding anatomy. Will the anatomical experiments still be of use in case no electro-physiological signatures of attention have been found?
    Reply: Section 3.4.3 has been extended to emphasize the value of the anatomical research, both in combination with the electro-physiological and pharmacological data as well as on its own.

- Datum antwoord: 01-02-2018
- Verstrekt(e) antwoord(en): De aanvrager heeft in de herziene versie de gevraagde aanvullende informatie gegeven.
  Via een schriftelijke evaluatieronde van de herziene aanvraag heeft de DEC geconcludeerd dat alle voor een advies benodigde informatie is geleverd. De gevraagde aanvullende informatie is verwerkt in de finale versie van de aanvraag die samen met het advies naar de CCD is gestuurd. Het DEC-advies is gebaseerd op de herziene versie.

10. Eventuele adviezen door experts (niet lid van de DEC): niet van toepassing
    - Aard expertise
    - Deskundigheid expert
    - Datum verzoek
    - Strekking van het verzoek
    - Datum expert advies
    - Advies expert

B. Beoordeling (adviesvraag en behandeling)

1. Is het project vergunningplichtig (dierproeven in de zin der wet)? Indien van toepassing, licht toe waarom het project niet vergunningplichtig is en of daar discussie over geweest is.
   Indien niet vergunningplichtig, ga verder met onderdeel E. Advies.
   Het project is vergunningplichtig.

2. De aanvraag betreft een nieuwe aanvraag / een wijziging op een bestaande vergunning.
   Nieuwe aanvraag – Zie A4

3. Is de DEC competent om hierover te adviseren?
   Ja

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

C. Beoordeling (inhoud)

1. Beoordeel of de aanvraag toetsbaar is en voldoende samenhang heeft *(Zie handreiking 'Invulling definitie project'; zie bijlage I voor toelichting en voorbeeld).*
   Deze aanvraag heeft als hoofddoelstelling: **Het begrijpen hoe het cerebellum aandachtsverschuiving controleert.** Aandacht is in belangrijke mate sturend voor ons gedrag maar hoe precies aandacht en verschuivingen in aandacht tot stand komt is niet goed begrepen. Om dit te onderzoeken zijn specifieke gedragstaken nodig waarbij een aandachtsverschuiving kan worden gekoppeld aan activiteitspatronen van de betrokken hersengebieden. Aandacht in de mens wordt in belangrijke mate bepaald door waar we precies naar kijken en daarom zijn visuele taken die aandacht vereisen bij uitstek geschikt om de onderliggende processen voor aandacht te bestuderen. Het is bekend dat signaalverwerking in de grote hersenen van groot belang is voor het tot stand komen van aandachtsverschuiving maar er zijn nu duidelijke aanwijzingen dat ook de kleine hersenen betrokken zijn. Met behulp van fMRI is een verhoogde activiteit gevonden in het cerebellum bij een aandachtsverschuivingen het betrokken circuit (DAN; dorsal attention network) is op die manier globaal in kaart gebracht. Het bestaan van een attentienetwerk in het cerebellum is zeer opmerkelijk aangezien er decennia lang vrijwel uitsluitend motorische functies aan het cerebellum werden toegeschreven. Echter de betrokkenheid van het cerebellum in aandachtsverschuiving is echter nooit causaal bewezen en dit project beoogt inzichten te verkrijgen hoe cerebellaire circuits betrokken zijn bij hogere cognitieve functies. Het feit dat patiënten met een verstoorde functie van het cerebellum een verminderd vermogen tot aandachtsverschuiving laten zien of andere aandachts-gerelateerde gedragsafwijkingen vertonen is een verdere aanwijzing van de betrokkenheid van het cerebellum bij deze taken en het geeft ook aan dat er een sociale relevantie is om dit circuit beter in kaart te brengen.

   Het onderzoeksproject bestaat uit twee samenhangende onderdelen/subdoelen om verschillende aspecten van dit complexe gedrag te onderzoeken door middel van gedragstaken waarbij aandachtverschuivingen worden onderzocht in een situatie waarbij een oogbeweging naar het nieuwe punt van aandacht wordt onderdrukt. Het eerste deel van het project is gericht op het nader in kaart brengen van de activiteit van de cerebellaire DAN neuronen tijdens deze aandachtverschuivingen. Ook zal worden getracht om

met lokale pharmaca (intracerebellaire injecties) de aandachtsverschuiving te beïnvloeden. In het tweede deel van het project zal worden bepaald wat de anatomische verbindingen zijn van de betrokken cerebellum gebieden met andere hersengebieden (tracing). Samen dragen de twee subdoelen bij aan het verkrijgen van meer inzichten in de hersenmechanismen die betrokken zijn bij aandachtsverschuiving en specifiek de rol die het cerebellum daarin speelt.

De DEC komt tot de conclusie dat de opbouw van de aanvraag in belangrijke mate overeen komt met voorbeeld 1 van de handreiking 'Invulling definitie project'. De twee subdoelen worden in sequentiële volgorde benaderd. Indien de elektrofysiologische afleidingen in combinatie met de farmacologische manipulaties duidelijke resultaten hebben opgeleverd die aannemelijk maken dat het afgeleide gebied in het cerebellum betrokken is bij aandachtsverschuiving zal de procedure voor de neuroanatomische tracing worden gestart (go/nogo). De DEC heeft hierover vragen gesteld en de herziene aanvraag is op dit punt verduidelijkt. Van het cerebellaire gebied dat wordt bestudeerd is bekend dat he betrokken is bij hogere cognitieve functies (Yeo et al., 2011). Dus zelfs als elektrofysiologische/farmacologische experimenten geen bruikbare data opleveren in relatie tot aandachtsverschuivingen in dit deel van het cerebellum zullen de tracingstudies toch waardevolle inzichten opleveren over de organisatie van de cognitieve cerebro-cerebellaire reciproke verbindingen en daarmee bijdragen aan de kennis over het cerebellum. De resultaten van de subdoelen samen leiden tot het bereiken van het hoofddoel. De twee subdoelen van het project zijn elk in voldoende mate uitgewerkt in de beschrijving van de strategie onder 3.4.1 van het voorstel en de onderlinge samenhang van de subdoelen en de dierproef in paragraaf 3.4.3. van het onderzoeksvoorstel. De aanvraag is naar de mening van de DEC te typeren als een project.

Uit de projectbeschrijving en bijlage is duidelijk welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welke procedures en welk ongerief individuele dieren zullen ondergaan. De DEC is er van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en dat er niet onnodig dieren gebruikt zullen worden.

Gezien het bovenstaande komt de DEC tot de conclusie dat de aanvraag voldoende samenhang heeft en daarmee toetsbaar is.

2. Signaleer of er mogelijk tegenstrijdige wetgeving is die het uitvoeren van de proef in de weg zou kunnen staan. Het gaat hier om wetgeving die gericht is op de gezondheid en welzijn van het dier of het voortbestaan van de soort (bijvoorbeeld Wet dieren en Wet Natuurbescherming).
Dit valt buiten de taakstelling van de DEC als beschreven in artikel 18a.2.b van de Wod. Naar deze specifieke informatie wordt in het huidige aanvraagformulier en de bijbehorende toelichting niet gevraagd en de aanvrager heeft deze informatie dan ook niet verstrekt. Het is voor de DEC daarom niet mogelijk om op dit punt een goed onderbouwde uitspraak te doen. De DEC wil erop wijzen dat mocht dit in sommige omstandigheden wel het geval zijn dat de CCD in een procedure voorziet waarin de aanvrager inzage krijgt en verweer kan voeren.
De DEC heeft echter geen redenen om te signaleren dat er mogelijk tegenstrijdige wetgeving is die het uitvoeren van de proef in de weg zou kunnen staan. Dit mede op basis van de afstemming van de aanvraag met de IvD en het feit dat de aanvrager hier geen melding van maakt.

3. Beoordeel of de in de projectaanvraag aangekruiste doelcategorie(ën) aansluit(en) bij de hoofddoelstelling. Nevendoelstellingen van beperkt belang hoeven niet te worden aangekruist in het projectvoorstel.
De doelcategorie –fundamenteel onderzoek- sluit aan bij de hoofddoelstelling.

Belangen en waarden

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

De verkregen fundamentele kennis zal uiteindelijk kunnen bijdragen aan inzichten in de hersenmechanismen waarmee aandacht wordt aangestuurd.

De aanvrager heeft duidelijk gemaakt wat de status van het onderzoeksveld is en inzicht gegeven in de stand van zaken in de wetenschap. De hersenmechanismen die aandacht sturen zijn grotendeels onbegrepen hoewel de betrokkenheid van een aantal hersengebieden, waaronder het cerebellum, is vastgesteld met fMRI studies in de mens. Deze techniek heeft echter onvoldoende temporele en spatiële resolutie om neuronale activiteitspatronen te meten op het niveau van individuele hersencellen en (lokale) populaties van hersencellen om zo tot nieuwe inzichten te komen. Invasieve elektrofysiologische methodes zijn daarom voor het bereiken van nieuwe inzichten essentieel. De aanvrager heeft duidelijk gemaakt dat dit project het eerste en enige project ter wereld is waarbij de rol van het cerebellair attentienetwerk op deze wijze zal worden onderzocht. De DEC is er daarom van overtuigd dat het directe doel van het project gerechtvaardigd is binnen de context van het onderzoeksveld.
Dit alles is voor de DEC reden om te concluderen dat de nieuwe kennis uiteindelijk zal bijdragen aan een vermeerdering van de inzichten in hoe het cerebellum betrokken is bij de hersenmechanismen waarmee aandachtsverschuiving wordt aangestuurd (directe doel) en dat het voorgestelde onderzoek met de voorgestelde aanpak haalbaar is (zie C6 en C7 voor een onderbouwing).

In de DEC is discussie geweest in hoeverre het onderzoek naar een tamelijk abstract begrip als aandachtsverschuiving sociale en maatschappelijke relevantie heeft temeer daar de gebruikte elektrofysiologische technieken niet in de mens toelaatbaar zijn. Naar aanleiding van een verzoek om aanvullende informatie op dit punt heeft de aanvrager dit nader uitgewerkt. Aandacht is van groot belang voor vrijwel alle cognitieve processen van de mens en is bepalend voor wat we bewust waarnemen en welke waarnemingen we wegfilteren, op welke stimuli we reageren en welke informatie we opslaan in ons geheugen. Verhulde aandachtsverschuiving (*covert attention shift*) is van groot belang binnen normaal sociaal gedrag met name van belang voor het herkennen van gezichtsuitdrukkingen. Een tekortkoming op dit gebied leidt tot het missen van informatie die van belang is voor soepel verlopende sociale interacties van het individu met andere individuen; dit speelt in autisme. Naar opvatting van de DEC heeft de aanvrager aangetoond dat dit project een cruciaal biologisch proces als onderwerp heeft en dat dat er een duidelijke noodzaak bestaat om de rol van het cerebellum daarin beter kennen.

Wat betreft het maatschappelijk doel: er is bewijs dat afwijkingen in het cerebellum resulteren diverse gedragsafwijkingen, zoals bovengenoemd autisme maar ook schizofrenie en dyslexie, die (ondermeer) gepaard gaan met verstoringen van de visuospatiële aandachtscontrole. De belangen voor deze patiëntengroepen en voor de maatschappij zijn naar de mening van de DEC aanwijsbaar en groot maar zullen pas op lange termijn tastbaar worden. Dit project is dan ook primair een fundamenteel-wetenschappelijk project waarbij de verkregen kennis van belang zal zijn om op termijn therapieën te ontwikkelen gebruikmakend van technieken die wel toelaatbaar zijn op de mens.

5. Benoem de belanghebbenden in het project en beschrijf voor elk van de belanghebbenden welke morele waarden in het geding zijn of bevorderd worden *(Zie Praktische handreiking ETK: Stap 2.B en tabel 1; zie bijlage I voor voorbeeld)*
De belangrijkste belanghebbenden in dit onderzoeksproject zijn:
> De maximaal 3 resusapen. De integriteit van de dieren zal op verschillende manieren worden aangetast. Door de gevangenschap zullen de dieren geen natuurlijk (groeps)gedrag kunnen ontplooien zoals onder meer natuurlijke omstandigheden. Het experiment

is gepland om te worden uitgevoerd op dieren die momenteel al zijn gehuisvest in de faciliteit en die al zijn uitgerust met een afleidkamertje. Een eventueel derde aap zal nieuw zijn en hij zal verschillende periodes van tijdelijk matig ongerief ondervinden ten gevolge van meerdere chirurgische ingrepen in de loop van de proef. Tijdens de uitvoering van de complexe gedragstaken zullen de dieren met hun hoofd worden vastgezet en zullen ze periodes van dorstgevoelens ondervinden. De dieren worden eenmalig getransporteerd naar een andere huisvesting om de injecties met virale vectoren voor de tracing mogelijk te maken. Op die manier wordt voldaan aan de huidigen eisen in het kader van de wet op het gebruik van genetisch gemodificeerde organismen (GGO). Na afloop van de proef zullen de dieren worden gedood.
> De bij de uitvoering van het project betrokken onderzoekers. Zij zullen een substantiele toename in kennis en vaardigheden verkrijgen. De carrièremogelijkheden van de onderzoekers zullen verbeteren door publicaties. Ook de kans op het behouden en verkrijgen van nieuwe onderzoeksmogelijkheden, veelal deels gebaseerd op een goede wetenschappelijke reputatie, zal toenemen. Deze waarden zijn naar opvatting van de DEC echter van gering gewicht in de ethische afweging.
> Onderzoekers in het veld van de neurobiologie. Dit onderzoek kent een grote fundamenteel-wetenschappelijke component en de te verwachte toename van de kennis over de rol van het cerebellum in aandachtsverschuivingen zal met peers en het publiek worden gedeeld via publicaties. De wetenschappelijke resultaten zijn van algemeen belang om de werking van het brein beter te begrijpen en zullen bijdragen aan een beter begrip van cerebellum-gerelateerde aandachtsstoornissen.
> De doelgroepen in de maatschappij. Aandacht is een fundamenteel biologisch proces en speelt een cruciale rol in vrijwel al onze gedragingen. Het is moeilijk precies aan te geven wat de reikwijdte van de verkregen kennis zal zijn. Wel zijn er specifieke patiëntenpopulaties aan te geven waar problemen met aandachtsverschuivingen veroorzaakt door afwijkingen in het cerebellum een rol spelen. Beter inzicht in hun problematiek is op zich een groot belang maar in hoeverre het project zal zich op de langere termijn zal vertalen in concrete resultaten die voor deze patiënten van belang zijn, is moeilijk te voorspellen.

6. Is er aanleiding voor de DEC om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken?
Nee. Het instituut werkt al geruime tijd met resusapen en wordt regelmatig door de NVWA geïnspecteerd.

Proefopzet en haalbaarheid

7. Beoordeel of de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven voldoende gewaarborgd zijn. Licht uw beoordeling toe. *(Zie Praktische handreiking ETK: Stap 1.C5).*
Gezien de trackrecord en het al verrichte vooronderzoek van de groep is de DEC ervan overtuigd dat de aanvrager over voldoende expertise en de geschikte infrastructuur beschikt om de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn te realiseren. Het voorstel bouwt op de ruime kennis en ervaring die is verkregen door de uitvoering van technisch sterk vergelijkbare experimenten met resusapen binnen de groep en op de nauwe samenwerking met een andere werkgroep binnen het instituut die ook met resusapen werkt. De benodigde kennis en ervaring in het gebruik van tracers en virale vectoren is aanwezig binnen de groep.

   De DEC is er van overtuigd dat de aanvrager voldoende expertise heeft om gedurende het project te kunnen blijven voldoen aan de 3V's.

8. Beoordeel of het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en of de

gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project. Licht uw beoordeling toe. *Zie Praktische handreiking ETK: Stap 1.C6).*

Het directe doel van het project is *het begrijpen van de rol van het cerebellum in de hersenmechanismen waarmee aandachtsverschuivingen tot stand komen*. Uit het reeds verrichtte onderzoek van de groep (en binnen een andere werkgroep of hetzelfde instituut) is gebleken dat resusapen goed te trainen zijn op taken die aandachtsverschuivingen betreffen en om informatie in een werkgeheugen te behouden voor uitgestelde oog- of arm bewegingen.

De DEC is van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen van het project en bij recente wetenschappelijke inzichten. De DEC acht het reëel om te veronderstellen dat op basis van de resultaten van de voorgenomen reeks experimenten beschreven in het project, veel nieuwe en aanvullende kennis zal worden verkregen. De keuze van het model en de keuze voor de resusaap als proefdier zijn gerechtvaardigd (zie C9).

Tijdens de uitvoering van het project zullen de in de aanvraag beschreven kaders, inclusief de kaders van ongerief, nauwgezet door de IvD bewaakt worden.

Welzijn dieren

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

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

De doelstellingen van het project vallen binnen de voorwaarden die door de Wod aan het gebruik van niet-menselijke primaten worden verbonden. De DEC heeft stilgestaan bij de vraag of de noodzaak voor de inzet van de resusaap als proefdier voldoende was onderbouwd. Voor de proeven is het essentieel dat de proefdieren in staat zijn om hun visuele aandacht te verleggen zonder gelijktijdige oogbeweging naar het object van aandacht; op die manier worden artefacten in de metingen als gevolg van de neuronale activiteit van de oogbeweging uitgesloten. Andere diersoorten (honden, katten, varkens, muizen, ratten) die als "lager" worden gekenschetst dan de niet-humane primaten zijn niet in staat de benodigde complexe visuele gedragstaken te verrichten. Ratten en muizen zijn niet geschikt omdat hun retina uniform gevoelig is. Hierdoor heeft een rat of muis, voor zover bekend, geen neurale circuits die het verleggen van de aandacht koppelen aan een uitgestelde oogbeweging waardoor de meest belangrijke uitleesparameter ontbreekt. Daarnaast wordt het verschuiven van aandacht bij knaagdieren vooral gestuurd aan de hand van multi-sensorische, tactiele en auditieve prikkels, en in veel mindere door visuele prikkels, en staat daarmee ver af van de sterk visueel gestuurde aan-

dacht in de mens en aap. Katten en fretten hebben weliswaar een vergelijkbare aansturing van de ogen als bij mensen en apen omdat ze frontaal-staande ogen hebben met een duidelijke fovea, maar het is niet mogelijk om deze dieren gedragstaken te leren met aandachtsverschuivingen zonder het maken van directe oogbeweging of om aandachtsverschuivingen te koppelen aan een uitgestelde motorische actie (oog- of armbeweging). De ontkoppeling van aandachtverschuiving en directe oogbewegingen is een typische eigenschap voor primaten. De grote overeenkomsten tussen de hersenanatomie van mens en de resusaap is een belangrijk onderdeel om uiteindelijk een translatie naar de mens te kunnen maken.

Er bestaan geen alternatieven op basis van (stam)cellijnen of computermodellen. Ook is het niet mogelijk om dit onderzoek in de mens uit te voeren (zie C14).

De DEC komt tot de conclusie dat voor het bereiken van de doelstelling de inzet van resusapen (*Macaca mulatta*) noodzakelijk is.

10. Geef aan of de dieren gehuisvest en verzorgd worden op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU. Indien niet aan deze minimale eisen kan worden voldaan, omdat het, om redenen van dierenwelzijn of diergezondheid of om wetenschappelijke redenen, noodzakelijk is hiervan af te wijken, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe.
De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn. De onderzoeksgroep doet veel moeite om de dieren sociaal te huisvesten en een verrijkte omgeving te bieden. Er is contact met een etholoog die adviezen geeft om een optimale selectie te maken voor de gepaarde huisvesting van de dieren en om binnen de mogelijkheden die er zijn de huisvestingcondities verder te verbeteren.
De wettelijke voorwaarden met betrekking tot het gebruik van GGOs in grote proefdieren vereisen dat de injecties met de virale vectoren worden uitgevoerd in een DM-III faciliteit. Het aanvragende instituut beschikt niet over een dergelijke faciliteit en daarom is een tijdelijke huisvesting elders noodzakelijk. De dieren blijven sociaal gehuisvest en worden gewend aan het verblijf in een transportkooi. De tijdelijke huisvesting op DM-III voldoet, volgens informatie van de IvD, aan de gestelde eisen.

Gedurende de proeven worden de dieren geconfronteerd met een gecontroleerde vloeistof opname. Alle procedures rondom de waterregulering zijn gedetailleerd en afdoende beschreven in de bijlage. In de optiek van de DEC is ook de noodzaak tot deze motiveringsmaatregel afdoende onderbouwd in bijlage 1 op pagina 6. Het gaat in dit project concentratie-vergende taken en het dier moet per meetsessie vele trials uitvoeren voor een valide statistiek. Het is bekend dat dit niet lukt zonder een lichte vorm van motivatie. Mogelijke alternatieven voor de waterregulering zijn beschreven en de uiteindelijke keuze voor waterregulering is afdoende gemotiveerd. De maatregelen om uitdroging te voorkomen zijn uitgebreid beschreven en zijn afdoende.
De DEC is tot de conclusie gekomen dat, in het kader van de proeven, waterregulering noodzakelijk is en de beste keuze om de dieren te motiveren zodat het mogelijk is om vele trials en zeer betrouwbare metingen en resultaten van vele cellen (30-100) uit één dier te verkrijgen. Dit gegeven draagt bij aan de opvatting dat voor wetenschappelijk solide conclusies twee dieren voldoende zijn.

11. Beoordeel of het cumulatieve ongerief als gevolg van de dierproeven voor elk dier realistisch is ingeschat en geclassificeerd. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C2)*.
De DEC heeft zich ervan verzekerd dat de aanvrager al het mogelijke zal doen om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk

te voorkomen. De onderzoekers hebben veel ervaring met dit onderzoek en zijn aantoonbaar voortdurend op zoek naar verbeteringen in huisvesting en, verfijningen van de chirurgische methoden en proefopzet om zo het ongerief te verminderen.

Het cumulatieve ongerief is door de onderzoekers ingeschat als matig. De DEC onderkent dat de classificering van het cumulatieve ongerief voor dit type dierproeven niet eenvoudig is; dit is eerder als dilemma genoemd. De proeven lopen over meerdere jaren en de dieren ondergaan in die periode verschillende chirurgische ingrepen met telkens periodes van maximaal 3-4 dagen matig ongerief gevolgd door herstel en daarnaast verschillende langere periodes waarin de aap gedragstaken moet uitvoeren onder milde druk van waterrestrictie (mild ongerief).
Het lijkt aannemelijk dat niet-humane primaten, met hun hoge sociale intelligentie, sterk reageren en anticiperen op terugkerende momenten van ongerief die zich gedurende een lange periode in hun leven voordoen. Het is echter de vraag of het cumulatieve ongerief om die reden hoger ingeschat moet worden dan het matige ongerief van de afzonderlijke handelingen en dus bijvoorbeeld ingeschat zou moeten worden als ernstig. De DEC meent dat waarnemingen bij eerdere experimenten van vergelijkbare aard bij de aanvragende instelling geen feiten en omstandigheden hebben opgeleverd die aan die opvatting concrete steun geven. De IvD is van mening dat, juist door het langzaam gewennen van de dieren aan de huisvesting en de gedragstaken, er een situatie ontstaat waarbij de dieren zeer coöperatief zijn en dat er geen sprake is van een grote psychische of fysieke belasting van de dieren tijdens de uitvoering van de gedragstaken. Recent gepubliceerde studies naar het cumulatief ongerief bij vergelijkbare experimenten bij andere instellingen geven steun aan de inschatting matig als niveau van het cumulatief ongerief (Pickard, 2013 en Prescott 2010). Bij het gebruik van andere diersoorten is het onwaarschijnlijk dat een dergelijke situatie zal kunnen worden gecreëerd en bij het gebruik van bijvoorbeeld honden, katten, fretten of varkens zou er eerder sprake zijn van ernstig ongerief.
Alles overwegende komt de DEC tot het oordeel dat een inschatting "cumulatief matig ongerief" op zich een goede weergave is van het ongerief in het licht van wat er in de Wet op de dierproeven bedoeld wordt met de term "ongerief" en classificatie "matig".

Dit alles neemt niet weg dat de DEC oog heeft voor het feit dat het zeer langdurige experimenten betreft, waarin de dieren gedurende langere periodes dagelijks worden ingezet in experimenten waarin zij met hun hoofd worden vastgezet, dorstgevoelens hebben en taken moeten verrichten waarmee zij vloeistof kunnen verdienen. Wellicht wennen zij hieraan en ervaren ze het – na die gewenning - niet meer als ongerief, maar feit is dat de dieren jarenlang in gevangenschap leven en hun leven volledig in het teken staat van deze experimenten. Het betreft dieren die zowel sociaal als psychologisch zeer complex zijn en dit stelt hoge eisen aan de omgeving en de sociale verbanden waarin de dieren leven. Er wordt weliswaar een reeks maatregelen genomen om de dieren een verrijkt leven te bieden maar dit is geen volwaardige vervanging voor een zelfstandig bestaan in groepsverband in natuurlijke omstandigheden. Het is echter lastig om te bepalen welke voor de dieren belangrijke natuurlijke gedragskenmerken ze worden ontzegd door de huisvesting en de experimenten en het is daarom moeilijk te bepalen in welke mate dit tot een aantasting van het welzijn en de integriteit van de dieren leidt.

In de aanvraag is er sprake van hergebruik. De dieren zijn al aanwezig op het instituut, zijn getraind en geïnstrumentaliseerd in het kader van een eerdere proef. De dieren voldoen aan de voorwaarde dat het dier, naast de headpost, nooit meer dan twee craniale implantaties zal krijgen. De IvD bevestigt dat tijdens de uitvoering van de vorige proef er zich geen (onvoorziene) omstandigheden hebben voorgedaan waarbij sprake was van ernstig ongerief.

Het voordeel van het geschetste hergebruik dat het totaal aantal dieren hierdoor zal verminderen en dat een extra periode van gewenning aan de faciliteit en trainingsprocedures zoals dat nodig is voor nieuwe dieren wordt voorkomen. Daar staat tegenover het

nadeel dat het ongerief voor een individueel dier zal toenemen en dat een dier langer in proef zit. De DEC volgt de motivering van de aanvragers m.b.t. het hergebruik voor de geplande experimenten en deelt de opvatting dat het cumulatieve ongerief voor het gehele traject als matig wordt geclassificeerd. Omdat het ongerief van het individuele dier groter wordt is zijn er ook overwegingen dat het gebruik van meer dieren toch te prefereren is. De DEC heeft dit in een eerder advies als dilemma benoemd.

12. Het uitvoeren van dierproeven zal naast het ongerief vaak gepaard gaan met aantasting van de integriteit van het dier. Beschrijf op welke wijze er sprake is van aantasting van integriteit. *(Zie Praktische handreiking ETK: Stap 1.C2). (zie bijlage I voor voorbeeld).*
De integriteit van de dieren zal op verschillende manieren worden aangetast. Door het leven in gevangenschap zullen de dieren zich niet kunnen ontplooien zoals in een zelfstandig bestaan in groepsverband in natuurlijke omstandigheden. De dieren worden weliswaar paarsgewijs gehuisvest, dit is een belangrijke verbetering t.o.v. solitaire huisvesting, maar het stelt de resusapen niet in staat om een normale sociale groepsstructuur te vormen. Ook met de talrijke "verrijkingen" die de dieren worden verstrekt, kan niet volledig worden voldaan aan hun sociale en psychische behoeften. Prikkels en omstandigheden waaraan de dieren in natuurlijke omstandigheden voldoening of welzijn zouden ontlenen worden zoveel mogelijk nagebootst of vervangen door andere prikkels en omstandigheden waarvan men aanneemt dat die een vergelijkbaar effect zullen hebben. Feit blijft echter dat het om een kunstmatige omgeving gaat waarin men onvermijdelijk op beperkingen stuit. Er worden de dieren ervaringen die hen voldoening en plezier geven onthouden (zie ook C11). Ten behoeve van de experimenten zijn diverse implantaten in en op het hoofd aangebracht. Tijdens de uitvoering van de complexe gedragstaken zullen de dieren met hun hoofd worden vastgezet en zullen ze periodes van dorstgevoelens ondervinden. Na afloop van de proef zullen de dieren worden gedood.

13. Beoordeel of de criteria voor humane eindpunten goed zijn gedefinieerd en of goed is ingeschat welk percentage dieren naar verwachting een humaan eindpunt zal bereiken. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3).*
De humane eindpunten zijn voor elk van de bijlagen dierproeven duidelijk gedefinieerd. De DEC is het met de aanvrager eens dat de kans klein is dat de dieren een humaan eindpunt zullen bereiken; dit op basis van ervaring. De aanvrager zal gedurende de gehele uitvoering van de proef het welzijn nauwgezet monitoren. De DEC is daarom van mening dat de aanvrager, indien de dieren toch een humaan eindpunt bereiken, tijdig in kan grijpen om onnodig lijden te voorkomen.

3V's

14. Beoordeel of de aanvrager voldoende aannemelijk heeft gemaakt dat er geen geschikte vervangingsalternatieven zijn. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3).*
De DEC is van mening dat de aanvrager voldoende aannemelijk heeft gemaakt dat er geen vervangingsalternatieven zijn. Het onderzoek heeft als doelstelling het begrijpen van de hersenmechanismen waarmee aandachtsverschuivingen wordt aangestuurd en hiervoor zijn complexe gedragstaken noodzakelijk. De voorgestelde proeven zijn niet mogelijk in de mens gezien de risico's van intracraniale injecties, het herhaald inbrengen van elektrodes en de kans op levensbedreigende infecties rondom het implantaat. Het gebruik van niet-invasieve methodes bij de mens zal niet resulteren in het behalen van het gestelde doel.
De zeer grote overeenkomsten in gedrag tussen de resusaap en de mens maakt dat het gebruik van deze soort de beste kans biedt op het realiseren van de doelstelling en het verhoogt de kans op een toekomstige translatie naar de mens.
De DEC is tot de conclusie gekomen dat voor het bereiken van de doelstelling de inzet

van apen noodzakelijk is en dat de aap (*Macaca mulatta*) de meest geschikte soort is. Zie ook paragraaf C9.

15. Beoordeel of het aantal te gebruiken dieren realistisch is ingeschat en of er een heldere strategie is om ervoor te zorgen dat tijdens het project met zo min mogelijk dieren wordt gewerkt waarmee een betrouwbaar resultaat kan worden verkregen. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
De DEC is van mening dat het maximale aantal te gebruiken dieren realistisch is geraamd en proportioneel is ten opzichte van de gekozen strategie en de looptijd. De aanvrager verwacht dat voor het project in totaal maximaal 3 dieren nodig zijn. Een aantal van 2 dieren per elektrofysiologische proef is gangbaar binnen in dit wetenschappelijke veld. Het gevraagde aantal van 2 dieren voor het project valt daar binnen. Echter het is niet uit te sluiten dat er zich in de loop van het project omstandigheden voordoen die de inzet van een extra dier noodzakelijk maken.

De criteria voor het gebruik van de maximaal twee extra dieren binnen het project zijn duidelijk beschreven in de aanvraag. De aanvrager heeft de DEC informatie verschaft over de kans dat het gebruik van een extra derde dier noodzakelijk zal blijken te zijn. Op basis van eigen ervaring wordt die kans op 22% geschat. De DEC onderschrijft de verwachting dat er een redelijke kans is dat het uiteindelijke aantal dieren op maximaal 3 zal uitkomen. In het geval de noodzaak ontstaat voor meer dan 3 dieren is een wijziging noodzakelijk.

Naar de mening van de DEC zijn de randvoorwaarden voldoende duidelijk beschreven om de IvD in staat te stellen de noodzaak van een extra dier te kunnen beoordelen.

Door het hergebruik wordt optimaal gebruik gemaakt van de proefdieren. Het gebruik van dieren vanuit andere proeven verhoogt het ongerief voor het individuele dier maar het vermindert het totaal aantal benodigde dieren voor het onderzoek van deze onderzoeksgroep als geheel. De DEC heeft dit in een eerder advies als een dilemma gesignaleerd.

16. Beoordeel of het project in overeenstemming is met de vereiste van verfijning van dierproeven en het project zodanig is opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
De DEC heeft zich ervan verzekerd dat de aanvrager al het mogelijke heeft gedaan om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen. De dieren worden geleidelijk blootgesteld aan nieuwe aspecten van de uitvoering van de gedragstaken. Een nauwgezette registratie van de hoeveelheid gedronken water vindt plaats om zo uitdroging en schade op de langere termijn te voorkomen. De chirurgische ingrepen worden door ervaren personeel uitgevoerd. In geval van een derde dier: de implantaten worden aangepast aan het dier dieren zodat het de kans op complicaties wordt verminderd. De dieren worden sociaal gehuisvest in tweetallen in een verrijkte omgeving. Zij blijven sociaal gehuisvest tijdens het verblijf buiten het Instituut i.v.m. de injecties met virale vectoren. De verwachting is dat humane eindpunten, om redenen van lijden van het dier, zelden zullen worden bereikt.

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

Dieren in voorraad gedood en bestemming dieren na afloop proef
De dieren worden in het kader van de proef gedood om het hersenweefsel te gebruiken

voor de reconstructie van de betrokken circuits. Zie voor details C19.

18. Geef aan of dieren van beide geslachten in gelijke mate ingezet zullen worden. Indien alleen dieren van één geslacht gebruikt worden, beoordeel of de aanvrager dat in voldoende mate wetenschappelijk heeft onderbouwd. *(Zie Praktische handreiking ETK: Stap 1.C3; zie bijlage I voor voorbeeld).*
De aanvrager gebruikt uitsluitend mannelijke dieren omdat mannelijke dieren zich makkelijker aanpassen aan de gepaarde huisvesting dan vrouwelijke dieren. Het gebruik van beide geslachten beperkt de mogelijkheden om ideale duo's te vormen voor de huisvesting wat van groot belang is voor het welzijn van de dieren.

19. Geef aan of dieren gedood worden in kader van het project (tijdens of na afloop van de dierproef). Indien dieren gedood worden, geef aan of en waarom dit noodzakelijk is voor het behalen van de doelstellingen van het project. Indien dieren gedood worden, geef aan of er een voor de diersoort passende dodingsmethode gebruikt wordt die vermeld staat in bijlage IV van richtlijn 2010/63/EU. Zo niet, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe. Indien van toepassing, geeft ook aan of er door de aanvrager ontheffing is aangevraagd *(Zie Praktische handreiking ETK: Stap 1.C3).*
In het kader van het onderzoek worden de dieren gedood om via histologische analyses inzicht te krijgen in de lokalisatie van de geïnjecteerde tracers om zo de bij aandachtsverschuivingen betrokken circuits in cerebellum en andere hersengebieden in kaart te brengen. Er wordt een voor de diersoort passende dodingsmethode gebruikt volgens bijlage IV van richtlijn 2010/63/EU (overdosis barbituraten gevolgd door cardiale perfusie met fixatief).

Indien niet-humane primaten, honden, katten of landbouwhuisdieren worden gedood om niet-wetenschappelijke redenen, is herplaatsing of hergebruik overwogen? Licht toe waarom dit wel/niet mogelijk is.
Hergebruik na afloop is niet mogelijk; de dieren worden in het kader van de proef gedood.

NTS

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

D. Ethische afweging

1. Benoem de centrale morele vraag *(Zie Praktische handreiking ETK: Stap 3.A).*
Rechtvaardigt het verkrijgen van fundamenteel-wetenschappelijke inzichten in de hersenmechanismen die (visuele) aandacht sturen, het cumulatieve matige ongerief dat maximaal 3 resusapen wordt aangedaan in het voorliggende project?

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

De volgende waarden/belangen zijn in het geding (zie onderdeel C5):
Waarden/belangen met betrekking tot de proefdieren: *maximaal matig nadeel*. Dit nadeel bestaat uit meerdere periodes van matig ongerief ten gevolge van het uitvoeren van de proef en uit langere periodes van licht ongerief door de uitvoering van complexe visuele gedragstaken en transport naar een ander instituut binnen Nederland. De dieren zullen onder de gegeven huisvestingscondities, ondanks de uitgebreide reeks maatregelen om het welzijn te verbeteren, niet hun gehele repertoire aan natuurlijk groepsgedrag tot uiting kunnen brengen.

Waarden/belangen van de onderzoekers: *veel voordeel*. Deze belangen bestaan voornamelijk uit het verbeteren van hun positie in het betrokken wetenschappelijke veld. Deze waarden zijn naar opvatting van de DEC echter van *relatief gering gewicht* voor de ethische afweging.

Waarden/belangen met betrekking tot de doelgroepen binnen het onderzoeksterrein van met name de neurobiologie: *veel voordeel*. Het voorgenomen project zal het fundamenteel wetenschappelijk inzicht in de neurobiologische mechanismen die aandachtsverschuivingen sturen naar verwachting substantieel vergroten. Met name de rol van het cerebellum is daarin tot op heden nog nooit met de benodigde technieken en resoluties onderzocht. De basale kennisvergroting wordt door de DEC gezien als een *zwaarwegend en groot voordeel*.

Waarden/belangen met betrekking tot de maatschappij (patiëntengroepen, *andere wetenschapsgebieden)*. De verkregen fundamentele kennis zal op termijn kunnen bijdragen aan verbeterde therapieën voor verschillende ziektebeelden (*autisme*, *schizofrenie* en *dyslexie)* waarbij de sturing van de aandacht (deels) door afwijkingen in het cerebellum is verstoord. Het is echter niet te verwachten dat op basis van de nieuw verworven kennis op korte termijn nieuwe of verbeterde therapieën kunnen worden opgesteld. *Op lange termijn is voordeel mogelijk maar de omvang van dit belang is op dit moment echter moeilijk in te schatten*. Dit belang is naar opvatting van de DEC daarom van *gering gewicht* voor de ethische afweging.

3. Beantwoord de centrale morele vraag. Maak voor het beantwoorden van deze vraag gebruik van bovenstaande afweging van morele waarden. Maak daarnaast gebruik van de volgende moreel relevante feiten: belang onderzoek (C4), kennis en kunde van betrokkenen (C7), haalbaarheid doelstellingen (C8), categorieën en herkomst dieren (C9), 3V's (C14-C18), ongerief (C10-13 en C19) en relevante wet en regelgeving (C2). Onderbouw hoe al deze elementen zijn meegewogen bij de beantwoording van de centrale morele vraag, zodanig dat het navolgbaar is zonder gedetailleerde kennis te hebben van het projectvoorstel *(Zie Praktische handreiking ETK: Stap 3.C; zie bijlage I voor voorbeeld)*.
De DEC is van mening dat de benoemde belangen van de wetenschap en samenleving in dit project zwaarder wegen dan de belangen/waarden van de proefdieren. De volgende overwegingen hebben bijgedragen tot deze conclusie:

   - Indien de doelstellingen bereikt worden, zal dit resulteren in een aanmerkelijke toename van de neurobiologische inzichten in de rol die het cerebellum vervult in de hersenmechanismen die (visuele) aandachtsverschuivingen sturen. Het is aannemelijk dat deze fundamenteel wetenschappelijk kennis zal bijdragen aan nieuwe inzichten in de oorzaken van bepaalde ziektebeelden in de mens. De DEC beschouwt vergroting van fundamentele kennis op dit onderzoeksterrein als een zwaarwegend belang.

   - Er zijn patiëntengroepen te definiëren waarbij een verstoring van de mechanismen die aandacht sturen een rol speelt. Het is het niet de verwachting dat de verkregen

inzichten op de korte termijn, d.w.z. binnen de periode van het project, zullen resulteren in nieuwe effectieven behandelingen. Op de lange termijn is dit echter niet uitgesloten; het belang van deze patiënten is daarom slechts in beperkte mate in onze afweging betrokken.

* De DEC is van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en recente wetenschappelijke inzichten en dat de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project.

* Het is aannemelijk dat de doelstellingen behaald zullen worden. Om dit doel te bereiken is het nodig resusapen te gebruiken. De onderzoekers doen er echter alles aan om het lijden van de dieren te beperken waardoor het uiteindelijk ongerief van elk individueel dier, naar verwachting, beperkt blijft tot maximaal matig ongerief. Zie voor een uitgebreide motivatie m.b.t. het ongerief onderdeel C11.

* De DEC is overtuigd van het belang van de wetenschappelijke doelstelling en het belang van de nieuwe kennis.

* De DEC is er van overtuigd dat de aanvrager voldoende kennis en kunde heeft om de doelstellingen te behalen en tijdens de uitvoering van het project te kunnen voldoen aan de 3V-beginselen.

* De DEC is van mening dat de aanvrager bij de uitvoering van het project alle mogelijke maatregelen treft om het ongerief van de dieren te beperken en het aantal dieren tot een minimum te beperken.

** Een positieve conclusie op een ieder van deze punten ziet de DEC als een noodzakelijke voorwaarde binnen de ethische afweging om te komen tot een positief besluit. Binnen dit project is naar inzicht van de DEC, op basis van alle verstrekte informatie, voldaan.*

Gezien bovenstaande overwegingen en conclusies is de DEC van opvatting dat het belang van de doelstelling, op de wijze zoals beschreven in deze projectaanvraag, het gebruik en het matig ongerief van proefdieren rechtvaardigt.

E. Advies

1. Advies aan de CCD
   - ■ De DEC adviseert de vergunning te verlenen voor maximaal 3 dieren.
   - ☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
     - ■ Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
     - ☐ Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
     - x Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten: Gegeven de informatie over de kans op de noodzaak van één extra dier is de DEC van mening dat de vergunning afgegeven moeten worden voor een maximum van 3 dieren. In het geval de noodzaak ontstaat om meer dan 3 dieren te gebruiken is een wijziging noodzakelijk.

   ~~☐ De DEC adviseert de vergunning niet te verlenen vanwege:~~
     ~~☐ De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:...~~
     ~~☐ De volgende doorslaggevende ethische bezwaren:...~~

~~☐ De volgende tekortkomingen in de aanvraag:...~~

2. Het uitgebrachte advies kan unaniem tot stand zijn gekomen dan wel gebaseerd zijn op een meerderheidsstandpunt in de DEC. Indien gebaseerd op een meerderheidsstandpunt, specificeer het minderheidsstandpunt op het niveau van verschillende belanghebbenden en de waarden die in het geding zijn *(Zie Praktische handreiking ETK: Stap 4.A; zie bijlage I voor voorbeeld).*
   Het advies is unaniem.

3. Omschrijf de knelpunten/dilemma's die naar voren zijn gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies zowel binnen als buiten de context van het project *(Zie Praktische handreiking ETK: Stap 4.B).*
   De DEC heeft vergelijkbaar met eerdere NHP projecten in eerder adviezen een tweetal dilemma's gesignaleerd: 1) het al dan niet toepassen van hergebruik en 2) de problematische inschatting van het niveau van cumulatief ongerief (zie onderdeel C11 van dit advies. Deze twee dilemma's zijn ook van toepassing binnen dit project en worden hier herhaald.

   1) Tijdens de uitvoering van de proef zullen de dieren nadat de proeven zijn voltooid opnieuw worden gebruikt voor vervolgexperimenten waarbij de dieren opnieuw moeten worden geopereerd. De voorwaarden voor het hergebruik zijn helder voor de DEC en IvD en het toezicht op de correcte invulling van het hergebruik zal naar verwachting geen problemen opleveren. Het voordeel van het hergebruik is dat het aantal benodigde dieren binnen de onderzoeksgroep op deze manier met maximaal de helft zal verminderen. Het nadeel is dat het ongerief voor een individueel dier zal toenemen maar dat een extra periode van gewenning aan de faciliteit en trainingsprocedures voor een nieuw dier wordt voorkomen. De DEC volgt de motivering van de aanvragers m.b.t. het gebruik van een dier voor vervolgexperimenten maar omdat het ongerief van het individuele dier groter wordt is het ook denkbaar dat het gebruik van meer dieren toch te prefereren is.

   2) De inschatting van het cumulatieve ongerief is bij proeven met resusapen van een duur van meerdere jaren is niet eenduidig. Binnen de duur van het experiment zijn er meerdere korte periodes van matig ongerief voornamelijk als gevolg van de chirurgische ingrepen. De ingrepen vinden plaats onder anesthesie en met pijnbestrijding en het matige ongerief wordt daarom voornamelijk veroorzaakt door desoriëntatie na het bijkomen uit de verdoving. Het interval tussen de ingrepen is dusdanig dat de dieren volledig herstellen. De DEC en de aanvrager schatten het cumulatief ongerief in als matig (zoals gemotiveerd in het advies); echter de volledige lijst van alle ingrepen en de langdurige gedragstaken dusdanig lang dat dat de effecten ervan op het dier niet met zekerheid zijn te voorspellen en dat dus niet valt uit te sluiten dat er onder omstandigheden toch ook ernstig cumulatief ongerief kan optreden.

Centrale Commissie Dierproeven **8**

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	De rol van de kleine hersenen in de aansturing van aandacht en de connectiviteit met de grote hersenen.
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	Aandacht, cognitie, cerebellum, oogbewegingen, anatomie

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☒ Fundamenteel onderzoek
- ☐ 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)

De hoeveelheid informatie die op elk moment via het netvlies binnenkomt in onze hersenen is zo groot dat het onmogelijk is om alle informatie bewust te verwerken. Daarnaast is een groot deel van de informatie niet relevant. Door onze aandacht te verplaatsen en te richten op specifieke delen van het gezichtsveld kunnen we een selectie maken tussen relevante en irrelevante visuele informatie.

Historisch heeft het onderzoek naar aandachtsverplaatsing zich voornamelijk gericht op de werking van de grote hersenen. Echter, recent onderzoek laat zien dat ook de kleine hersenen een belangrijke rol spelen in aandachtsverplaatsing. Zo laten verschillende studies zien dat bepaalde delen van de kleine hersenen actief worden als mensen een visuele aandachtstaak

		uitvoeren. Deze studies maken gebruik van functionele MRI, een techniek om de activiteit van hersenen bij proefpersonen in beeld te brengen. fMRI is echter te langzaam en mist de precisie om vast te stellen wat de activiteit van individuele zenuwcellen is tijdens aandachtsverplaatsingen. Door gebruik te maken van proefdieren, kunnen we wel technieken gebruiken voor het meten van individuele cellen zodat we een beter begrip krijgen van de "berekeningen" die de kleine hersenen uitvoeren tijdens het maken van aandachtsverplaatsingen. De hersenen functioneren als een groot netwerk. Om te begrijpen hoe de hersenen bepaalde taken uitvoeren is het daarom essentieel om de verbindingen tussen individuele cellen te kennen. Door na afloop van de metingen kleurstoffen te injecteren in de kleine hersenen kunnen we de verbindingen met de grote hersenen die een rol spelen in visuele aandachtsverplaatsingen in kaart brengen.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Aandacht is een van de hoekstenen van onze denkprocessen en daarmee een essentieel onderdeel van vrijwel elk aspect van menselijk gedrag. Beter begrip over hoe de hersenen aandachtsverplaatsingen sturen draagt bij tot een begrip de rol van een verstoorde aansturing van aandachtsprocessen bij neurologische aandoeningen, zoals autisme, schizofrenie en dyslexie. Kennis over de normale werking van aandachtsprocessen geeft inzicht in de symptomen van deze ziekten en is daarmee, op langere termijn, van belang bij toekomstige behandelingen voor deze ziekten.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Voor dit onderzoek gebruiken we maximaal 3 resusapen.
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Negatieve gevolgen voor het welzijn van proefdieren zijn 1) ongerief als gevolg van de verschillende chirurgische ingrepen, verricht onder volledige anesthesie en afdoende pijnbestrijding, 2) het ondervinden van stress tijdens het aanleren en uitvoeren van de taken; dit gebeurt stap voor stap om stress te verminderen.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Voor de resusapen zal het ongerief matig zijn, omdat voor de proeven operaties onder anesthesie nodig zijn. Dit niveau van matig ongerief is van korte duur. De rest van de tijd dat een aap in de proef zit, is het ongerief licht.
3.6	Wat is de bestemming van de dieren na afloop?	Een belangrijk onderdeel van dit onderzoek is het ophelderen van de verbindingen tussen zenuwcellen die de visuele aandacht sturen in de kleine hersenen. Daarom zullen de apen aan het einde van de proeven gedood worden zodat de zenuwverbindingen bestudeerd kunnen worden onder de microscoop.

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	Studies met niet-invasieve technieken bij mensen verstrekken waardevolle informatie over de hersengebieden die betrokken zijn bij de controle van aandacht. Echter, deze technieken missen de precisie om de activiteit van de zenuwcellen te kunnen bestuderen. Dat gaat slechts door de activiteit van hersencellen te meten met elektroden in het brein. Dit vergt langdurig invasief onderzoek dat niet bij mensen wordt uitgevoerd. Voor dit onderzoek is een geschikte proefdiersoort nodig met een hersenanatomie die vergelijkbaar is met die van de mens en die aandachtstaken kan uitvoeren.

gebruikt kunnen worden.	Knaagdieren kunnen enkele eenvoudige cognitieve taken uitvoeren, maar ze hebben geen mechanisme voor het verplaatsen van visuele aandacht, waardoor het onmogelijk is aandachtsprocessen in knaagdieren te bestuderen. Katten zijn ook vaak gebruikt in studies over het visuele systeem, maar ook zij kunnen niet getraind worden om de aandacht te verschuiven zonder een oogbeweging te maken. Aandachtsprocessen in resusapen lijken op die in de mens en ook de anatomie van de betrokken hersengebieden is vergelijkbaar. Er is daarom bij dit onderzoek geen alternatief voor de resusaap beschikbaar.
4.2 **Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Het is onze ervaring dat we betrouwbare resultaten verkrijgen met twee of drie apen per experiment. Het voorgestelde aantal apen is het minimale aantal dat nodig is om statistisch betrouwbare resultaten te verkrijgen.
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.	Alle procedures (inclusief de huisvesting van de resusapen) zijn erop gericht om het ongerief voor de dieren zoveel mogelijk te beperken. Er is veel bekend over de kleine hersenen van de resusaap en de aap is de enige proefdiersoort die aandacht aanstuurt op een manier die goed overeenkomt met de aandachtsturing 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.	1) We beperken de hoeveelheid stress zoveel mogelijk door de apen geleidelijk te laten wennen aan alle aspecten van de gedragstaken. Dit is ook van belang voor het welslagen van de experimenten, daar gestreste dieren niet of nauwelijks zullen participeren. 2) De dieren worden getraind met een waterrestrictieregime waarbij zij de benodigde dagelijkse hoeveelheid vocht als beloning krijgen tijdens de training. We hanteren een zorgvuldig protocol om negatieve effecten van de gecontroleerde vloeistofopname te voorkomen. In onze ervaring leidt dit protocol tot gering ongerief en zijn er geen negatieve gevolgen voor de gezondheid. 3) Alle operaties worden uitgevoerd onder anesthesie door personen die goed zijn opgeleid en ruime ervaring hebben. Na de operaties worden pijnstillers gebruikt om postoperatieve pijn te voorkomen. 4) De dieren worden sociaal gehuisvest in tweetallen in een verrijkte omgeving, om zo het ongerief van het leven in een kooi te beperken en om de verstandelijke vermogens te verbeteren. Het doden van de dieren aan het einde van de proeven vindt plaats onder volledige anesthesie.

5 In te vullen door de CCD

Publicatie datum	

Beoordeling achteraf	
Andere opmerkingen	

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'.	10.2.g
1.2	Provide the name of the licenced establishment.	10.2.g
1.3	Provide the title of the project.	Understanding control of covert attention by the cerebellum.

2 Categories

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

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ 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.

Outline of the project

With this project we want to increase our understanding on the role of the cerebellum in

cognition, specifically in visual attention shifts. The focus will be on two sub-aims. 1. Deciphering the neuronal firing patterns that take place in individual cells when a non-human primate is performing an attention task. 2. Unravelling the connections that the cerebellum makes to the attention related areas in the cerebral cortex, to gain a systems level understanding of the attention network. For this project two, maximally three, rhesus monkeys are required.

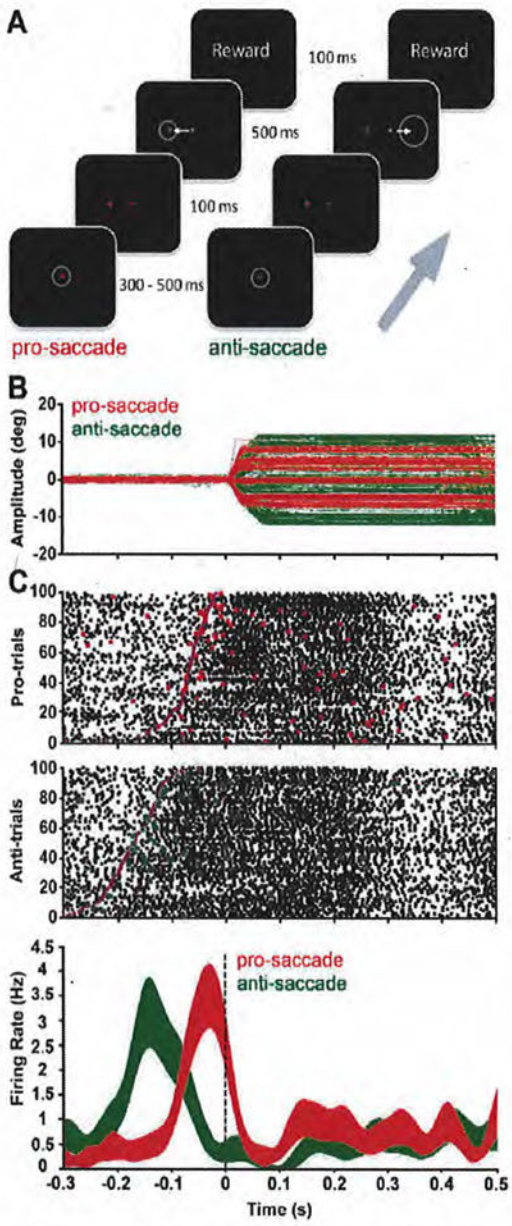
3.1 Background

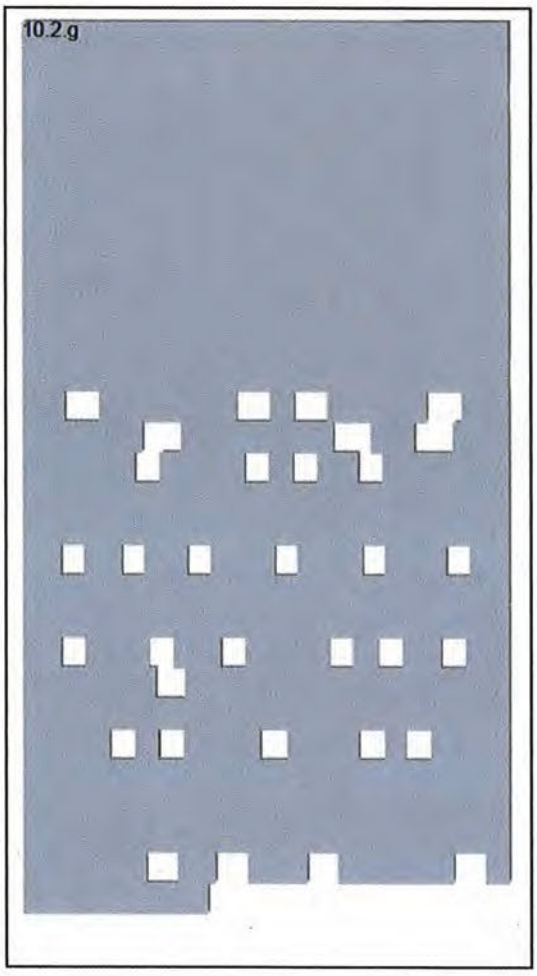
Introduction: visual attention shifts

For adequate visual perception and visually-guided decision making, it is essential to separate relevant from irrelevant visual information. Our eyes are continuously confronted with an overwhelming amount of information, yet we understand our visual world without any apparent effort. How does the brain parse relevant and discard irrelevant information? Visual attention refers to the cognitive operations that allow us to efficiently perform this selection. Visual attention shifts can be differentiated into overt and covert orienting. Overt orienting refers to the process of moving the eyes to the object of interest. Covert orienting attention on the other hand is the process of mentally shifting attention in the visual field *without* moving the eyes. When making overt attention shifts, the fovea, which is the area of the retina with the highest visual acuity, is moved to an object of interest. The fovea is an extremely small (< 1%) part of the surface area of the retina, thus, a whole world exists outside the scope of the fovea. When shifting attention covertly to stimuli, the visual processing of stimuli outside the fovea is enhanced, giving it more weight in what we experience as a visual precept. Covert attention shifts are faster than overt attention shifts and decrease reaction times for overt attention shifts (Posner, 1980). The social meaning of eye position is a well-studied subject under psychologists. Assessing what a person's intentions; dispositions and beliefs are and even their willingness to initiate a conversation can in some situations all be read from their gaze (Cary, 1978; Emery, 2000; Laidlaw, Rothwell and Kingstone, 2016). Monkeys show similar behavior; like humans they can read many social cues from gaze position and facial expressions, however looking a monkey in the eye is highly threatening for them and can even evoke a fight. This illustrates why it is often advantageous to conceal where one looks, while still extracting scene information by means of covert attention shifts.

Involvement of cerebellum in attention shifts

Understanding how the brain controls attention is one of the challenges of contemporary neuroscience. Most research on this topic focuses on the control of attention by the cerebral cortex. However, recently multiple lines of evidence demonstrate that the cerebellum also plays an important role in the control of covert attention shifts. The idea that the cerebellum does anything other than motor behavior is relatively new (Buckner, 2013) and the involvement of the cerebellum in the deployment of visual attention is a highly understudied field. In our previous research we have shown that the cerebellum contributes to complex decision-making. For example, primates were presented with a visual stimulus and taught to make a saccade to or away from a visual target. Monkeys had to direct their eyes to a red or green visual target in the center of the screen. Based on the color of the visual target they had to decide to either make a saccade to a second, off-center visual target (pro-saccade), or to look in the opposite direction (anti-saccade, fig 1A). During this task activity was recorded from individual cells in the cerebellum, and a marked change in activity occurred during the decision phase just prior to initiating the eye movement (fig 1B,C) (10.2.e en 10.2.g Studies of patients with natural lesions to the cerebellum (e.g. hemorrhage, tumor) have demonstrated impairments in covert attention tasks (Le, Pardo and Hu, 1998; Townsend *et al.*, 1999; Christopher L Striemer *et al.*, 2015). The cerebellum is subdivided into 10 lobules (I-X), multiple fMRI studies have shown increased activity in the lateral part of lobules VII and VIII of cerebellum when human subjects perform covert attention shifts (Allen, 1997; Tomlinson *et al.*, 2014; C. L. Striemer *et al.*, 2015). Resting state MRI (rsMRI), a proxy for connectivity between brain regions, indicates that these lobules are connected to the **dorsal attention network (DAN, figure 2)**, which controls top-down attention, with both overt (i.e. eye movements) and covert shifts of attention (Corbetta and Shulman, 2002). The cortical areas of the DAN: the frontal eye fields and intraparietal sulcus, are functionally connected to the cerebellum.





A study by Brissenden et al. (2016) elegantly combined fMRI studies with anatomical evidence of connectivity between the DAN and the cerebellum. They measured the fMRI activity of the whole cerebellum during a covert attention task. In another experiment, they measured cerebellar connection strength to DAN with rsMRI. They found a robust linear correlation between cerebellar activity during the attention task and connection strength to the DAN, implying that the cerebellum is an integral part of the dorsal attention network. The limitation of the aforementioned studies is that they do not show how the cerebellum participates in the control of covert attention.

Anatomy of the connections between the DAN and the cerebellum

As famous scientists Crick & Koch stated "In biology, if seeking to understand function, it is usually a

good idea to study structure." The cerebellum is massively interconnected with the cerebral cortex. However, little is known about how connectivity between the DAN and the cerebellum is involved in the control of non-motor behavior. With the exception of anatomy based on MRI data there are only a few studies that look at connectivity between the cerebral cortex and cerebellum (Kelly and Strick, 2003; Glickstein, Sultan and Voogd, 2011; Lu, Miyachi and Takada, 2012; Voogd, 2014). Unfortunately, the spatial resolution of MRI based anatomy is inadequate to understand how the areas function as a network. Thus, alternative approaches must be used, for example the injection of anatomical tracers which are taken up by neurons. After injection, the tracer is transported through the cell and thereby highlights axonal pathways with single cell resolution. An important advantage of tracing individual cells is that it's possible to inject the dyes exactly where task-related neurons have been found in physiological experiments, thus highlighting unique connections of an area with a specific function. The currently existing neuro-anatomical tracing studies highlight the pathways from the cerebral cortex through the pons to the cerebellum: the cortico-ponto-cerebellar pathway (figure 2). However, there is another extensive input to the cerebellum from the cerebral cortex, through the mesodiencephalic junction (MDJ) in the brainstem; the cortico-mesodiencephalic-cerebellar pathway. This input is well charácterized in rodents in relation to motor behavior (10.2.e en 10.2.g), however no studies have been undertaken when it comes to non-motor behavior.

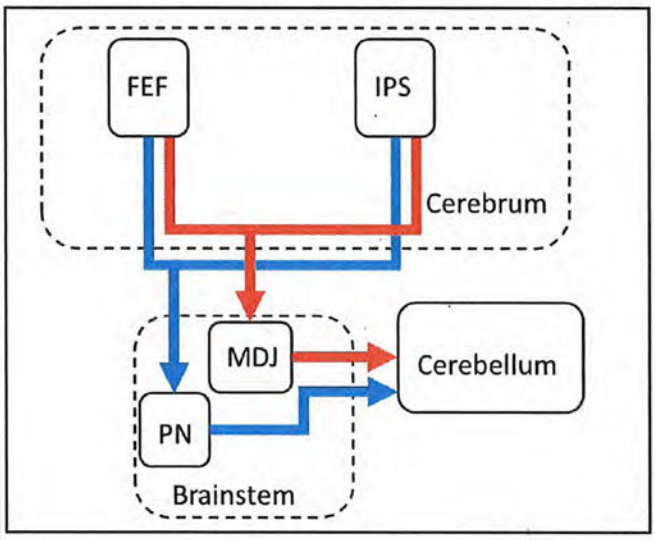


Figure 2. Dorsal attention network with proposed connections between cerebral and cerebellar parts. Blue arrows represent cortico-ponto-cerebellar pathway; orange arrows represent the proposed cortico-mesodiencephalic-cerebellar pathway. Abbreviations: FEF frontal eye fields, IPS intraparietal sulcus, MDJ mesodiencepahlic junction, PN pontine nuclei

Open questions on the role of the cerebellum in attention shifts

There is strong evidence that suggests a role for the cerebellum in the control of covert attention shifts. The evidence that exists however is of correlative nature, thus it is necessary to identify if there is causal involvement of the cerebellum during covert attention shifts. Experiments are therefore required to determine the exact nature of cerebellar contributions to attentional shifts and what type of firing patterns are involved. Lastly, it is clear from previous studies that there is strong connectivity between the areas of cerebellum and the cerebral cortex that control attention. These connections are relayed in the brainstem. However, it is unknown which of the specific areas functions as relay between the cerebral DAN and the cerebellum. Therefore, to gain understanding of how the brain control attention as a whole, the exact connectivity between the cerebral and cerebellar areas that control attention needs to be elucidated.

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10.2.e en 10.2.g

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 objective of this project is to understand how the cerebellum controls covert attention shifts in cognitive tasks. We will focus on: (1) how the cerebellum controls covert attention shifts at the level of single neuron activity and (2) how the cerebellum is anatomically connected to the dorsal attention network.

The main aim of this application is to understand how the cerebellum controls covert attention shifts. To reach our aim, we need animals trained on a task in which they will have to make covert attention shifts. Both these sub-aims will be addressed in experiments with macaque monkeys. Experimental procedures required to reach both sub-aims are described in a single animal procedure 3.4.4.1.

Sub-aim 1: To determine how the cerebellum controls covert attention shifts and through what (single neuron) coding strategies.

The control of attention shifts by the cerebellum on a single neuron level has never been investigated, although there is a vast amount of evidence from MRI, lesion studies and studies on cerebellar disorders that all indicate cerebellar involvement in this type of behavior. The high spatial temporal precision of micro-electrode recordings can teach us what input-output correlations individual cerebellar neurons make to execute trained behaviors. Switching off small parts of the network with reversible pharmacological interventions will show us whether a causal role of lobules VII and VIII exists in the control of attention shifts.

Sub-aim 2: To determine the connectivity between the lateral cerebellum and attention controlling networks in the cerebral cortex.

The cerebellum has modular connections that form closed-long-distance loops, in which a descending arm from neocortex reaches the cerebellum (via pontine nuclei) and an ascending arm projects from the cerebellar nuclei to the neocortex (via thalamus). The cerebellum receives another descending input from the cerebral cortex through the mesodiencephalic junction in the brainstem. DAN to cerebellum connectivity has never been studied in primates on a cellular level, therefore we don't know which descending pathway is utilized by the attention network. Brain regions are never solitary responsible for the performance of a behavior. Accordingly, to understand how the brain accomplishes a behavior, especially as complex as visual attention, it is essential to understand the connectivity of the entire network. Using anatomical tracers, we will elucidate the pathways between the cerebral DAN and the cerebellum. Furthermore, immunohistochemistry will be performed to determine which neurotransmitters are used by these cells to provide information on the excitatory or inhibitory character of the projecting neurons.

Feasibility of the projects

The experiments described in this protocol are based on techniques that have been established in our lab, both in rodents as well as primates. We have extensive experience in training animals on complex tasks, performing single neuron recordings and high-level data analysis and modelling. In addition, the experimental design is based on tasks that are known to provide robust behavioral outcomes (Ignashchenkova *et al.*, 2004). State-of-the-art equipment is present at the lab to perform the described measurements with the highest degree of accuracy. At the same institute, another research group is performing similar research on non-human primates for many years with great success. With them we share cutting edge animal facilities and operating rooms. As a close collaborator we often discuss the progress of our research, possible improvements to the tasks, caretaking and well-being of the animals. If the current researcher is not able to finish the project, the research will be continued by a different researcher.

3.3 Relevance

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

Scientific Relevance

The topic of representation and control of attention in the brain is one which receives constant attention in both popular and scientific media. The possibility that the cerebellum plays a role in attention shifts has been discussed for more than 20 years, however it still remains to be causally proven (Allen, 1997). In recent years evidence has emerged on cerebellar involvement in many cognitive functions in health (Schmahmann, 2001; Moore *et al.*, 2017; Wagner *et al.*, 2017) and disease (Wang, Kloth and Badura, 2014a). To formulate adequate hypotheses of how the cerebellum contributes to cognitive processes, it is essential to know the physiology and anatomy of these networks and how they are connected to other attention controlling regions in the brain. Since the first publication on covert attention in the cerebellum by means of fMRI (Allen, 1997), no attempts have been made to investigate how attention is controlled at a cellular level. The current study will provide the very first insights in exactly how cerebellar circuits

Social relevance

Covert orienting attention is impaired in many groups of psychiatric patients including those suffering from, autism, schizophrenia, dyslexia (Courchesne *et al.*, 1994; Maruff *et al.*, 1995).
These disorders are all strongly associated to the cerebellum through behavioral, genetic and cellular biomarkers. (Courchesne *et al.*, 1988; Pernet *et al.*, 2009; Wang, Kloth and Badura, 2014b) For instance, deficits in visual attention shifts have been shown in both children and adults with autism spectrum disorders (ASD) (Townsend *et al.*, 1999; Landry and Bryson, 2004) Covert attention plays an important role in the social cognition we exercise in our day to day social interactions. Attention is reflexively shifted toward faces, from which we can read all sorts of information on the basis of facial expression or gaze direction. Failing to make covert attention shifts can thus result in the loss of many important socials cues, which is the case in individuals suffering from autism (Frith and Frith, 2008; Pfeiffer, Vogeley and Schilbach, 2013). Studies of patients with damage to the cerebellum (e.g. through hemorrhage, tumor) have demonstrated impairments in covert attention tasks. Also, fMRI studies demonstrate a clear relationship between covert attention shifts and cerebellar activity (Brissenden *et al.*, 2016).
Impairments in *autism* are strongly associated with the cerebellum:

1. Damage to the cerebellum at birth leads to a risk ratio greater than 40 (Limperopoulos *et al.*, 2007). This high ratio is consistent with the fact that cognitive and affective deficits often have been shown to arise after cerebellar injury:
2. Cerebellar injury during a premature birth is followed by autism-like symptoms (Bolduc *et al.*, 2012).
3. After birth, pediatric insult to the vermis leads to cognitive and affective deficits (Riva and Giorgi, 2000).
4. Neuro-anatomical studies show strong correlation between autism spectrum disorders (ASD) phenotype and cerebellar insult and/or hypoplasia (Abell *et al.*, 1999).
5. In rats, midline cerebellar lesions at P10 cause perseveration and social disruption in the adult (Bobée *et al.*, 2000). In mice Purkinje cell-specific disruption of the tuberous sclerosis gene *Tsc1* produces autism-like deficits in mice (Tsai *et al.*, 2012).

These findings suggest that the cerebellum plays a specific early-life role in autism, most probably in guiding the development of core social capabilities. They are also consistent with the fact that the cerebellum is among the most frequently disrupted brain regions in autistic patients. Adult ASD cerebella show alterations in their olivary, deep nuclear and Purkinje cells (Palmen *et al.*, 2004). The cerebellum also shows gray and white matter abnormalities (Courchese *et al.*, 2005). Furthermore, recent studies have used aggregated gene expression patterns to ask when and where ASD genes are expressed. ASD susceptibility genes show a high degree of co-expression with one another in the mouse and human brain, allowing the identification of specific gene networks (Menashe *et al.*, 2013). These networks show that one of the regions where genetically driven programs can go off-track is the cerebellar cortex.

The current study contributes to the understanding how the cerebellum performs cognitive functions in a healthy state. Knowledge of the physiology of these processes enables the invention of therapies aimed at normalizing the diseased brains state instead of treating symptoms. . Lastly, the insights from this study may aid more guided experiments with techniques that are compatible for use in humans and provide target locations for interventions for instance through new non-invasive techniques such as transcranial magnetic stimulations.

3.4 Research strategy

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

General Research strategy

To explore how the cerebellum controls attention, we will perform neurophysiological recordings from individual cells (single unit recordings to detect spiking activity), transiently suppress the activity of neurons in parts of the network, and inject neuro-anatomical tracers. The combination of these techniques will provide better insight in how the cerebellar network that controls attention functions.

Sub-aim 1: To investigate how the cerebellum controls covert attention shifts and through what (single neuron) coding strategies.
Primates will be trained on a task in which they have to covertly shift their attention to a target in the peripheral visual field without making eye movements. Execution of attention shifts can be read from the presence of micro-saccades, pupil dilation and shorter reaction times for saccades. Measurements on the activity of single neurons will provide us with a direct measure of the input output correlations that cerebellum makes to achieve this behavior. Small variations in the task, such as variation in the amplitude of the stimulus, variable delays between task parameters or the addition of a correct or incorrect cue, will provide further insight in the dynamics of the network during the execution of the behavior. The experiments will be performed in soundproof rooms so to prevent distractions during task performance. Also, the task will be repeated over tens to hundreds of trials to establish a robust cellular response, this will have the advantage of averaging out any transient cofounding factors. To establish a causal link between the cerebellum and covert attention shifts specific temporary blockers that are locally active will be administrated. The covert attention stimuli will be presented on both sides of the visual field, therefore when pharmacologically suppressing activity in one side of the cerebellum we will expect uni-lateral impairments of task performance where the contralateral side can function as control.

Sub-aim 2: To investigate the connectivity between the lateral cerebellum and attention controlling networks in the cerebral cortex.
We aim to elucidate the anatomical connections between the cerebral dorsal attention network(DAN) (i.e. frontal eye field and intraparietal sulcus) and the cerebellar areas that are necessary for this task. After the neurophysiological experiments are finished, we will inject viral trans-synaptic neuroanatomical tracers in the cerebellar cortex. The advantage of viral tracers is that they can cross connected synapses. There are at least two synapses between the cerebellar cortex and the cerebral cortex. This poses a problem for non-viral tracers, as they cannot cross synapses and thus can only be used to elucidate part of the network. In contrast to MRI based anatomy, anatomical tracers provide the exact paths that individual neurons follow to communicate with each other. We will inject tracers that label cells which have connection towards and away from the cerebellum. The analysis will be carried out on brain tissue of the animals after they are euthanized and perfused with fixative. By looking at both routes, a network level understanding will be obtained of the circuits controlling attention throughout the brain instead of in isolated brain regions. This information could then be applied in interventions that treat some of the disorders mentioned in the social relevance section.

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.

Basic habituation, chair training, and surgeries (AP 3.4.4.1)
A *newly acquired* animal follows a highly standardized training program, from habituating the monkey to the primate facility up to the point where they can perform visual tasks in daily training sessions. These initial stages have been carefully optimized and are performed or overseen by the well-trained technicians and animal caretakers of the facility. This standardization ensures a very high success rate acclimatizing monkeys to the facility and the training routine. Monkeys are obtained from the national primate center or, in exceptional cases, from licensed importers if there are no appropriate animals available at the primate center. They are socially housed in the facility, typically in pairs.
After habituation to the new environment, monkeys are trained to move into a primate chair that allows them to be transported comfortably from their home-cage to the experimental set-up. Once the animal can sit quietly in the chair for periods of >1 hour the animal undergoes a surgery to implant a head-post. The head-post is a small rod attached to the skull of the animal, it is used to fixate the head of the monkey during training and later electrophysiological recordings. This is an essential step because we train the monkeys to control their eye position and these measurements are only possible if the monkey's head is fixed.

After recovery from the head-post surgery, the animal is habituated to having his head fixated in the chair. Most monkeys adapt very quickly to this step. The monkey then starts daily training sessions in which it acquires juice rewards for performing simple eye-movement or hand-movement based tasks.

Initially the tasks are very simple, such as directing his gaze ('fixating') on a large dot on a computer screen for a few hundred milliseconds. During this process, the animal will be placed on a controlled fluid uptake regime. Gradually the difficulty of the tasks is increased by making the dot smaller until the animal can fixate, then make guided eye-movements towards visual targets or hand-movements after the presentation of a 'go' cue. At this stage, the animal is ready to be trained on the experimental tasks.

Neural recordings and inactivation during attention demanding task performance (AP 3.4.4.1)

The animals will be trained on a task where they have to shift their attention without moving their eyes. During the task, measurements on single cells will be made with acute micro-electrodes inserted for the duration of the recordings via the implanted recording chamber over the cerebellum. The cellular activity can be correlated with different task parameters to gain insight in what kind of activity patterns are used by the cerebellum that are related to the attentional shifts. In other experiments, the performance on the task will be studied when activity in lobules VII and VIII of the cerebellum is suppressed through application of pharmacological agents, such as the selective GABA agonist muscimol. Dosage and volumes injected will be adopted from the literature, since many studies exist that apply these agents during task performance in the cerebellum. These agents, depending on the specific drug, work on the time scale from minutes to hours to a full day. This permits studying task performance before, during and after application, giving insight in whether the cerebellum is necessary to perform the task.

Neuro-anatomy of connectivity between the dorsal attention network and cerebellum (AP 3.4.4.1)

To elucidate the anatomical connections between the cortical DAN and the cerebellum, viral trans-synaptic tracers will be injected in the cerebellar cortex. These tracers can be injected through the recording chamber in the same way as the pharmacological agents. For the injection of viral tracers bio-safety level 3 facilities are necessary. Since these facilities are not present at the Institute, the animals will be transported by car to another Institute. After the injection, the animals will remain there to recover for a few days during which the tracer will spread through the nerve cells, after which the animals will be euthanized and transcardially perfused to fixate the tissue. Subsequent staining of tissue sections will give a precise picture of the neuronal connections between the DAN and the cerebellum.

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.

The aim of this project is to understand how visuospatial attention is controlled by the cerebellum and the connections to the cortical attention related areas. This approach requires a species with comparable neuroanatomy to the human and the ability to perform attentional tasks. Rodents are able to perform some simple cognitive tasks, however there are critical differences between the functioning of the rodent and primate visual system which makes rodents unsuitable for this study. Cats have also often been used in basic visual studies, but these animals cannot be trained to perform covert attentional tasks.

Two animals from other previously finished experiments can be re-used in this proposal. They have been used in the saccade/anti-saccade experiments described before, however in the current proposal these animals will be used for a different scientific question. In the saccade/anti-saccades experiments a similar experimental approach was used as proposed here, including single unit recordings from the cerebellum. For these experiments, both animals have had recording chambers implanted, which are still in good shape and in the right spot for the experiments planned in the current proposal. The animals are already habituated to the facility and used to performing cognitively demanding visual tasks, thus we known that the animals can learn and robustly perform the new task.. The cumulative discomfort of the procedures these animals have gone through is classified as moderate. Re-using the animals for these experiments leads to a total reduction of the animals used and eliminates the discomfort new animals would face during acclimatization and surgeries. Re-use also eliminates the delays associated with the surgeries and habituation required when a new monkey is used.
We would like to stress that given that there are no alternative animal models for these experiments monkeys would be used regardless of the presence or absence of already trained individuals. Therefore, the presence of the animals at the facility is not the reason for the submission of this proposal. If one of the currently trained animals has to be withdrawn from the study (see appendix 3.4.4.1), a new monkey will be purchased.

The first stage in the project is to train the animals on the new task. This will take 6 months. When sufficient task performance has been reached (consistent correct responses above chance level), neuronal recordings will start (24-30 months). After successful recordings and correlations between electrophysiological data and behavior have been established, inactivation experiments will be performed to establish a causal link between this area and the task (6 months). This order will ensure that we first collect enough baseline data make a comparison with the inactivation experiments so that we can detect even subtle changes in both behavior and electrophysiological readings.
When robust results have been obtained in the electrophysiology and pharmacology experiments the neuro-anatomical tracer experiments will be done. Since the neuroanatomical tracer experiments are terminal they will be done last. Anatomical information on cerebro-cerebellar loops relating to cognition is extremely valuable, because it can only be obtained on a cellular level through injection of neuro-anatomical tracers. Therefore, these data cannot be obtained from postmortem slices of human brains, and also not from lower animals since they don't possess the same level of cognitive neuro-development as human or primates. The area in which the neuro-anatomical tracers will be injected is known for its involvement with higher cognitive functions(Yeo *et al.*, 2011). Hence, even without the association of attention shifts with this part of the cerebellum the anatomical data will still provide valuable insights in the organization of cognitive cerebro-cerebellar loops and will likely be the foundation of much further research. The use of neuro-anatomical tracers requires the animals to be euthanized and their brains need to be extracted for sectioning and histology (2 months). The described experiments are likely to last less than the 5 years, the maximum duration of a CCD project. However, when the possibility of an animal dropping out (~ 12 months delay), or implants needing replacement (~ 6 months delay) are taken into consideration significant delays can be expected.

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	**Cerebellar measurements of neural activity and tracer injections** training – electrophysiological recordings – intracranial injections of pharmacological agents – tracer injections - perfusion
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'.	10.2.g
1.2	Provide the name of the licenced establishment.	10.2.g
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 Type of animal procedure: **Measurements of neural activity in cognitive tasks and tracer injections.**

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.

The procedures described in this appendix concern 1) measurements of neural activity in a task investigating the deployment of attention and 2) injections of neuroanatomical tracers followed by ex vivo analysis of the connections

Monkeys will be trained to perform complex cognitive tasks in which they must attend visual stimuli while and keeping their eyes fixed on a different location on the screen. They will be trained to make behavioural choices using an eye-movements. The animals will receive implants (recording chambers), which will allow us to access the cerebellum with electrodes for recording neural activity while the animal performs the task. We will use tungsten electrodes to record the activity of individual neurons. We will use pharmacology to locally alter the neuronal activity.

The primary outcome parameters are:

i) The behaviour of the animal. The animal's accuracy and reaction time on the behavioural tasks will be recorded. We also investigate how behaviour depends on alterations of neuronal activity.

ii) Neural activity recorded from the electrodes. All electrode types described here can record single- and multi-unit activity and well as the local field potential. We will examine the link between neural activity recorded in the cerebellum and the behaviour of the monkey.

iii) Neuroanatomical connections revealed by the injected tracers.

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

Two animals from previous experiments performed by our lab can be reused in this proposal. They have been used in the saccade/anti-saccade experiments described in the project proposal. In those experiments a similar setup was used as proposed here, including single unit recordings from the cerebellum. The recording chamber implanted for these experiments is still in good shape, and in the right spot to reach lobules VII & VIII, which we will target in the experiments proposed in the current proposal. The animals also are already habituated to the facility and to performing cognitively demanding visual tasks.

Only if one of the present two animals has to be withdrawn from the study, a new monkey will be purchased, the animal will be acquired and acclimatized to the primate facility (left part of figure 1). They will undergo structural anatomical scans to guide the design of the surgical implants. The animal will be implanted with a head-post, which allows the head of the monkey to be fixed in the experimental set-up. The animal will be placed on a controlled fluid regime and trained on basic tasks such as fixating on small regions of a computer screen and making eye-movements to visual targets. After reaching high levels of performance on these basic tasks the animal will then be trained on more complex attention-demanding tasks. Once trained on these tasks the animal will be implanted with a recording chamber targeting the cerebellum. The recording chamber will be used with electrodes for neurophysiological recordings and gives access to the cerebellum for pharmacological experiments. After recovery from the implantation, we will then begin electrophysiological experiments in which we record neural activity from the electrodes while the monkey performs the task.

The flow of animals through this animal procedure is described in the figure below:

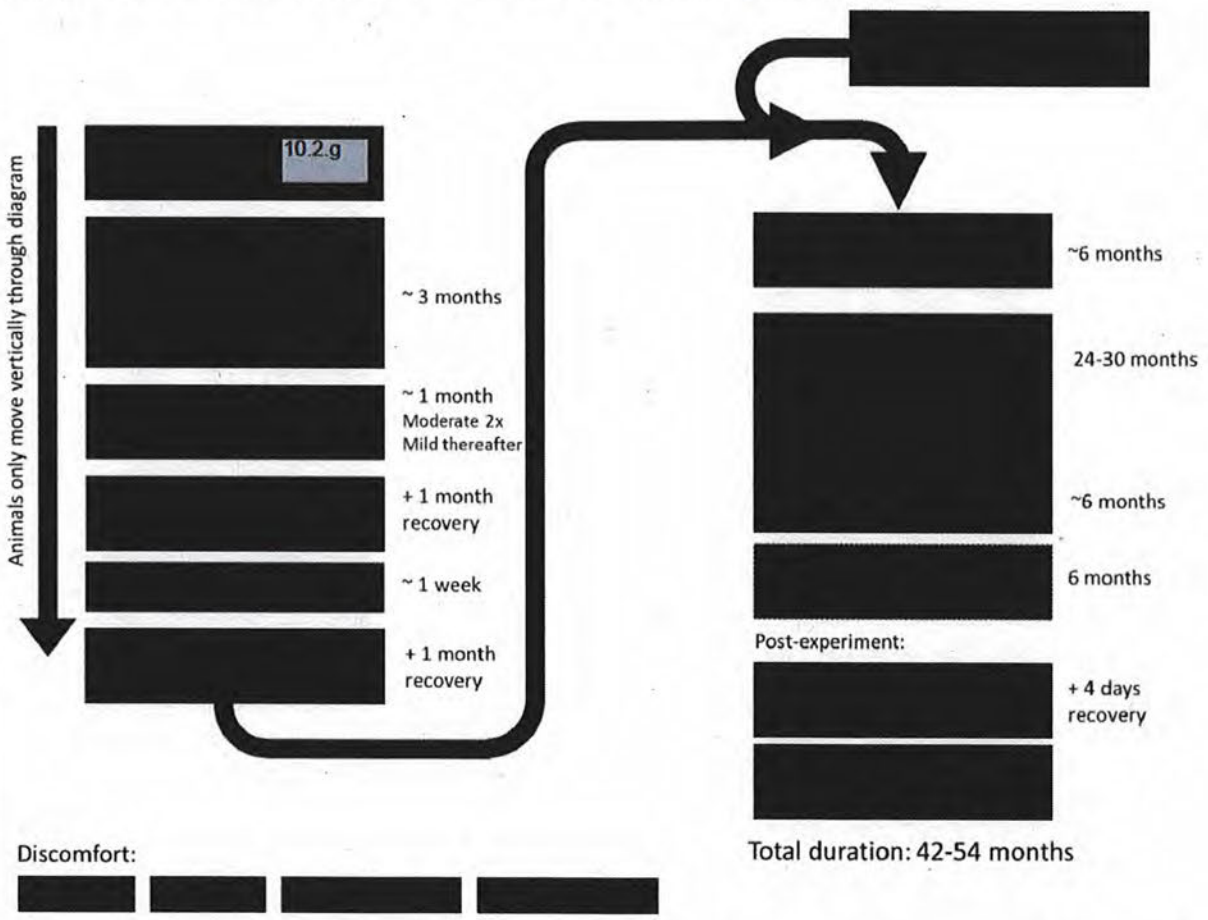

Figure 1 – A flow diagram outlining the steps of procedure 3.4.4.1. *The left part of the scheme is only applicable for a newly acquired monkey.* ***2 of our monkeys will be reused from previous***

experiments, for them only the right part is applicable. *CFU refers to periods in which the fluid uptake of the animal is controlled.*

The following procedures are described in the current appendix (3.4.4.1)

1. **Acquisition and housing**
2. **Acclimatization**
3. **CT scanning**
4. **MRI scanning**
5. **Chair training**
6. **First surgical procedure: Head-post implantation**
7. **Head-fixation training**
8. **Controlled fluid uptake**
9. **Behavioural training on basic tasks**
10. **Behavioural training on complex tasks**
11. **Second surgical procedure: Chamber implantation**
12. **Recordings sessions**
13. **Pharmacological Interventions**
14. **Removing tissue above the dura**
15. **Third surgical procedure: implant restorations**
16. **Annual health check**
17. **Transport and temporary housing at DM3 level**
18. **Injection of viral vector**
19. **Perfusion**

Note that step 1 thought 9 are only applicable if a monkey drops out and has to be replaced by a newly acquired monkey.

1. **Acquisition and housing (only for a new animal)**

A new monkey will be obtained from a licensed breeding facility. In all cases, we will first try to obtain a new animal from a national primate centre. Only under exceptional circumstances (no monkeys available at the primate centre) we will get them from a licensed importer. Monkeys will be housed in the primate facility of our institute. All animals are male and typically between 3-5 years of age when they arrive. This is the age at which young male monkeys typically leave their social group. We typically acquire two cage-mates together and these are then pair-housed for 3-4 weeks in a cage in isolation from the other monkeys (for quarantine reasons). When the results of viral and bacteriological tests are negative we can, if desired, pair these monkeys with established members of the group. We consult an ethologist from a national primate centre who advises us on appropriate pairings. This can be desirable to form stable pairings or larger groups (if the social character of the animals allows it). Our facility contains large cages and the monkeys will have access to a floor-to-ceiling play cage, which allows them to climb and swing. The play cage also contains a 'look-out' platform where the monkeys can view other monkeys in the facility. The environment will be enriched with toys (e.g. boxes filled with nuts or sweets, which the monkeys can fiddle out) and access to natural daylight. A TV screen shall be running in front of the cages during the day. A logbook will be maintained individually for each of the monkeys, carefully monitoring their general appearance, their eating behaviour, weight, and the performance during the training sessions.

2. **Acclimatization (only for a new animal)**

Discomfort: Mild or none

The monkeys will be adapted to the animal housing facility and the staff. This includes but is not limited to an initial period in which the animal will be housed with a partner, will receive daily food treats from the staff, and will have access to toys in his cage and television. Previously acquired monkeys in the facility have successfully undergone this period of adaptation and interact well with the staff and do not exhibit signs of stress due to their environment. During this period, the monkey will receive a CT and MRI scan (see below). During one of these procedures the monkey will also be fitted with a collar, which

is later used for guiding the monkey into a primate chair.

3. CT scanning (only for a new animal)

Discomfort: Mild (recovery from brief anaesthesia), once per animal (unless complications require re-assessment)

A CT scan may be obtained to allow 3D models of the monkey's skull to be constructed. These are used to custom design surgical implants which perfectly fit the skull of the animal. The monkey is anesthetized in its home cage, and then transferred to the CT scanner. The scanning procedure lasts less than 5 minutes. The monkey is then returned to his home-cage, and he is allowed to recover from anaesthesia. The total duration of the procedure is approximately 30 minutes. When there are no complications, this procedure will only take place once per animal. Occasionally, in the event that an implant comes loose, we may perform a further CT scan to assess the state of the underlying bone.

4. MRI scanning (only for a new animal)

Discomfort: Mild (recovery from brief anaesthesia), once per animal (unless complications require re-assessment)

Structural MRI scans are obtained to check whether the brain has any anatomical anomalies, and to localize specific brain structures and plan surgical implants. The monkey is anesthetized in its home cage and then transferred to the MRI facility. The anatomical scan lasts approximately 15-20 minutes, after which the monkey is returned to his home-cage, and allowed to recover. If there are no complications, this procedure will only need to be performed once per animal.

5. Chair training (only for a new animal)

Discomfort: Moderate the first 1-2 times, none after this.

The collar will be used to gently guide the monkey into the primate chair. Food and liquid rewards will be used in order to classically condition the monkey to enter the chair. Once learnt, the monkeys usually get into the primate chair voluntarily and rapidly. Once this behaviour is acquired, the animal will initially be rewarded with fruit or fruit juice for sitting quietly in the chair for short periods of time. The head of the monkey is not fixated at this stage. The time spent in the chair will gradually be increased as the animal becomes ever more comfortable and will be adjusted according to the animal's behavioural reaction.

6. First surgical procedure: Head-post implantation (only for a new animal)

Discomfort: Moderate for 1-2 days, becoming mild for 2-3 days.

All surgeries are performed in the purpose-built primate operation room within the primate facility of our institute. Specialist anaesthesia equipment is available and the surgeries are performed by trained staff. In order to head fix our monkeys during training, a head-post is attached to the skull. After induction of anaesthesia, an incision is made in the skin, and the skin is gently pulled aside, exposing the area of the skull above the cortex. The head-post is attached and the skin is sutured closed. Analgesics are given during the surgery. The duration of the procedure is approximately 1-2 hours.

At the end of the surgery, the animal is monitored and kept warm while waking up. Additional analgesics are given during the recovery period. Following the surgery, training will be discontinued for at least four weeks so that the animal may recuperate. After this time, the head-post is solidly fixed to the animal's skull.

7. Head-fixation training (only for a new animal)

Discomfort: Mild (but decreasing after 1-2 times), approximately 1 week

The animal will receive food and juice rewards for sitting quietly in the chair with their head fixated via the implanted head-post. The amount of time spent fixated in the chair will increase progressively and will be modulated according to the behavioural reaction of the individual animal. Once the animal quietly sits in the chair with his head fixed for a sufficient period of time (0.5 hours), the animal will begin training on the basic experimental tasks. This step usually takes about a week, with the discomfort of the procedure being mild for the first one or two times that the animal is fixated, and lower after this.

8. Controlled fluid uptake (all animals)

Discomfort: Mild

To motivate the animals to work their access to fluid is controlled. The main reason why we use controlled fluid uptake is that we need to obtain a sufficiently large number of trials per session, for two reasons. First, we need reliable measures of the animals' perception which demands a large number of trials. Second, we obtain a larger number of trials to study the activity of neurons. The activity of neurons is inherently stochastic, i.e. the responses of a cell to repetitions of the same stimulus are variable, a stochasticity that is inherent to proper brain function. Controlled fluid access is by the far the most common method to motivate animals to perform cognitive tasks. We note that only healthy and cooperative monkeys that are at ease will perform these tasks in which they make eye or arm movements. Alternative methods have been explored as described by a workgroup for the British NC3R centre (National Center for Replacement, Refinement and Reduction of Animals in Research) (Prescott et al., 2010). These alternatives comprise 1) positive reinforcement with fruit juice, without controlled fluid uptake; 2) food-based reinforcement; 3) electrical stimulation of reward centres in the brain. Reinforcement with juice in the absence of controlled fluid uptake works well in the early stages of training when training sessions are short and tasks simple, but it is insufficient to motivate the animal to perform more difficult tasks or a larger number of trials. Food reinforcement with treats like raisins or peanuts is used in our lab to reward an animal for compliant behaviour (for instance, for coming to the correct compartment of the cage to interact with a researcher). It can also be used as reinforcement for short and simple tasks at the start of training. However, animals satiate quickly when rewarded with food and chewing movements cause artefacts in our recordings, which makes this type of reinforcement unsuitable for sessions that require many trials and precise recording of neural activity. Electrical stimulation of the reward centres involves an extra surgery with the accompanying risks of complications and direct electrical stimulation of the basal ganglia may interact with the neural processes that are the main focus of this application.

Controlled fluid uptake is thus the only viable method available to obtain sufficient numbers of trials to be able to reliably measure behaviour and neural activity. We implement controlled fluid uptake in a gradual fashion that adapts the level of fluid control to the behaviour of each individual. We begin with positive reinforcement using fruit juice without any controlled fluid uptake. We only use fluid control regimes if the animal is not sufficiently motivated to perform the task with no fluid control. We gradually introduce the fluid control with the aim to have the animals drink as much fluid as possible and the fluid control is only made stronger if necessary. Nevertheless, in the majority of animals it is necessary to restrict access to fluid to some level to obtain enough trials on the complex behavioural tasks described in the application. The amount of fluid control is individually determined for each monkey and we always begin by training animals without any fluid control. Most animals require some level of restricted access to fluid to motivate to perform behavioural tasks, and almost all animals require restricted access to motivate them to work on complex tasks. Our aim is to allow the animal to drink fluid during performance on the behavioural task until they are satiated. This is achieved by:

- The difficulty of the task is adjusted on each day so that the animal is able to receive fluid at a high rate, motivating him to work for more trials, and drink more fluid in total.
- The rate of fluid delivery is slowly increased during a training session to ensure that the animals drinks throughout the session.
- If the training session has to be aborted, for example due to a technical fault, then the animal receives fluid equivalent to the average intake during a training session.
- We investigate the preferences of each animal for particular rewards e.g. apple juice, different types of fruit syrup, or water, and use a reward that is appealing to the animal

We take a number of measures to prevent dehydration:

- The monkeys always receive a minimum of 100ml of fluid each day. If this amount is not reached during the training session, it is supplemented.

- Averaged over a three-day period, the animals must receive a minimum of 35 ml per kilogram metabolic weight per day, this number is based on recommendations by the British N3CR (National Center for the Replacement, Refinement, and Reduction of Animals in Research) (Prescott et al., 2010) and the primate facility of UC Davis (2001). For example, a 10kg animal with a metabolic weight of $10^{0.75}$ kg must receive a minimum of: $10^{0.75}$ x 35 = 197ml of fluid per day, averaged over the previous three days. If this average is not achieved, the animal is supplemented with fluid. This is a minimum amount and the animals typically receive much more fluid than this.
- Fluid intake, both received during training and supplemented in the cage, is logged in an electronic system accessible by researchers, caretakers and inspectors.
- The animal is provided with fruit after the training session, the liquid content of the fruit is not counted towards the minimum amount.
- During breaks in the training schedule of more than one day (e.g. weekends) the monkey receives a full water bottle of at least 700ml, animals over 15kg receive an extra bottle. If the break is only one day, then the animal receives an amount of fluid equal to what it would typically receive during a training session.
- While the animal is under fluid control, the researchers and animal caretakers monitor its appearance and behaviour carefully every day, with checks by the animal caretakers during the weekend. We weigh the monkey before and after training and compare the weight to the average weight during the last week. The weight is also checked over longer intervals to prevent a slow loss of weight. We check the monkey for any signs of dehydration such as reduced skin tension, sunken eyes, either increased or reduced activity, and dry faeces. If any of these welfare criteria is abnormal, the monkey is taken out of training and provided with ad libitum access to fluid until it has recovered. In that case, the Animal Welfare Body will be informed so that they can check the animal. These criteria (weight, fluid consumed per day) are logged in an electronic system for each monkey so that the history is accessible.
- The animal receives a non-working period once every 9 weeks (on average over a year). During this period the animal is not trained and receives a full bottle each day (>700ml).

The British NC3R centre investigated in 2010 the use of controlled fluid regimes in brain research with macaque monkeys (Prescott et al., 2010). Their conclusion was that, when a controlled-fluid protocol is carefully applied and monitored, there are no negative consequences for the health of the animal. Follow-up research from the University of Newcastle (Gray et al., 2016) showed that controlled fluid uptake for 7 days per week did not lead to abnormal blood values or signs of dehydration. Another study (Hage et al., 2014) analysed a broad range of behaviours over several months during fluid control and found no evidence for alterations in behaviour, which indicates that the animals' wellbeing can be stably ensured during training sessions with a proper protocol. Indeed, from their general appearance, it is very difficult, if not impossible, to distinguish between monkeys under fluid control and monkeys with ad libitum access to water. Furthermore, the animals are seen regularly by a veterinarian to inspect their general condition, and we investigate measures of kidney function during the yearly check-ups. We have never obtained indications of impaired kidney function. Hence, our own experience is in accordance with the literature, which indicates that a careful protocol of controlled fluid uptake is a safe and effective manner to motivate animals to perform the required cognitive tasks.

Gray et al., 2016. Physiological, Behavioral, and Scientific Impact of Different Fluid Control Protocols in the Rhesus Macaque (Macaca mulatta). eNeuro 3(4).

Hage, S.R., Ott, T., Eiselt, A.-K., Jacob, S.N., Nieder, A., 2014. Ethograms indicate stable well-being during prolonged training phases in rhesus monkeys used in neurophysiological research. Lab. Anim. 48, 82–87.

Prescott M.J., Brown V.J., Flecknell P.A., Gaffan D, Garrod K, et al., 2010. Refinement of the use of food and fluid control as motivational tools for macaques used in behavioural neuroscience research: Report of a Working Group of the NC3Rs. J. Neurosci. Methods 193, 167–88

University of California Davis, 2001. Policy statement: water restriction in rhesus behaviour studies. UC Davis Office of

Environmental Health and Safety.

9. Behavioural training on basic tasks (only for a new animal)

Discomfort: Mild

To motivate monkeys to perform their task, they are placed on a fluid control regime (described below). During training, the monkey is presented with sensory stimuli and responds with an eye movement and/or hand movement. We use positive reinforcement to train the animal, correct responses are

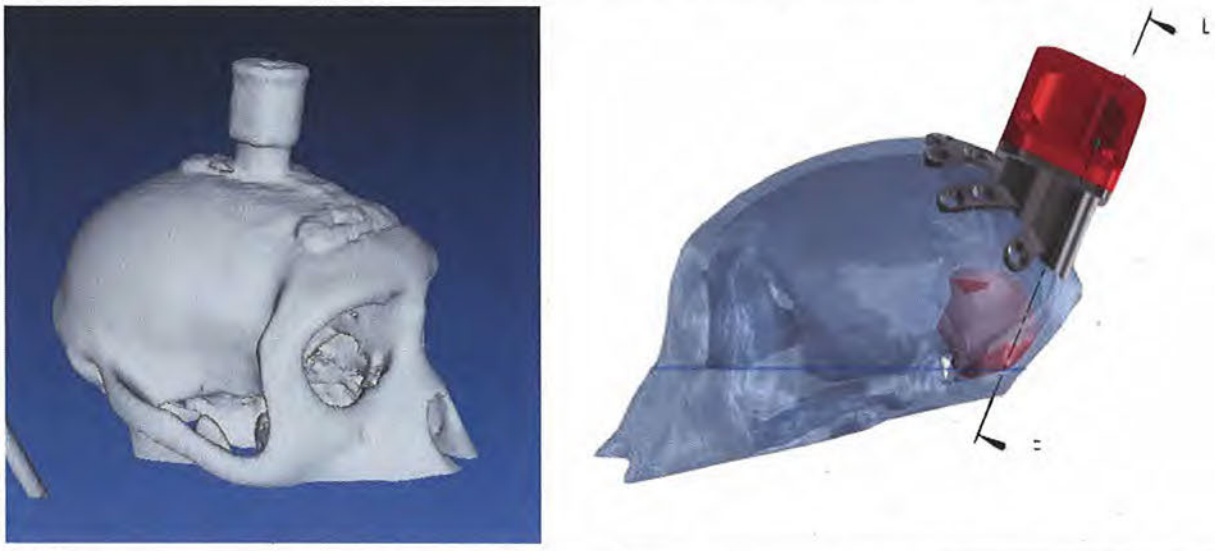

Figure 2. Chamber and head-post. *The image to the left shows a 3D reconstruction of the skull (viewed from the right) from a CT scan of a monkey who had been implanted with a head-post several months prior to the CT. The head-post had become very well integrated into the skull. These 3D models are also used to design recording chamber. The second image shows a recoding chamber over the cerebellum with in red angular adapter so the lateral parts of the cerebellum can be reached on both sides. The black line represents the path of an electrode reaching the cerebellum (red).*

followed by a fluid reward and the animal are allowed to work until satiated. The size of the reward is individually determined and is adapted throughout the training session to ensure that the monkeys remain motivated to work. No negative reinforcement is used, incorrect trials are typically followed by a lack of reward, and in some cases a small 'timeout' (5-10 s) may be given. As the monkeys learn the paradigm and their performance increases, we gradually make the task more challenging. Task difficulty is adjusted to ensure that the monkeys are able to obtain their full fluid ration during the training session. During the training periods, animal are typically in the setup 5 days per week, 1-4 h per day (typical is 2-3 h). Training on the initial tasks takes between 2-6 months depending on the monkey. An example of a training task is to have the monkey direct their gaze to a very small region of a computer screen for 1 s (known as 'fixation'). The difficulty of the task slowly increased by gradually decreasing the size of the area that the animal must fixate upon while slowly increasing the duration of the fixation. At the end of the training period the animal is expected to be able to fixate in a 1^0 diameter window for at least 400 ms and perform delayed saccade-tasks. A saccade is a very rapid eye-movement that monkeys and humans make approximately 3 times per second to direct the eyes to objects of interest. In a delayed-saccade task the animal must wait for a 'go' cue (e.g. the fixation dot changes colour) before making his eye movement. The duration of this training period varies from 1 to 6 months depending on the aptitude of the monkey. In our experience, all monkeys are able to learn these tasks within 6 months.

10. Behavioural training on complex tasks (all animals)

Discomfort: Mild

The goal of the animal experiments described here is to understand how the deployment of attention is controlled by the cerebellum. To this end, we train the monkeys on tasks in which they have to attend to a particular visually presented object and report an aspect of the stimulus. Examples of such tasks include:

Attention tasks:

The animals will be trained to covertly shift their attention to a target and subsequently show that they have observed the target by making a saccade to a certain direction to obtain a reward. First, the animal has to fixate at a point in the centre of the screen. Then, a target appears in the periphery of the visual field. During the presentation of the target, the animal has to keep fixation on the central point and covertly shift its attention to the target to determine the direction to which he has to make a saccade to obtain the reward (fig 3). Then there is a variable delay before the final saccade target is presented. The variable delay facilitates the decoupling of cellular activity related to the attention stimulus and the subsequent saccade. In the experiment, the amplitude of the attention shift is varied by changing the distance between the target and the fixation point. This permits establishment of correlations between neuronal activity and individual task parameters, such as target direction and amplitude. These correlations can determine what part of the behaviour is executed by a particular cell. Regression models will be applied to resolve the nature of the correlations between neural activity and behaviour for instance linear regression between firing frequency and target amplitude. The difficulty of the task is slowly increased over days and the presentation of the stimulus is varied along several dimensions (e.g. size and position of the stimulus) to ensure that the monkey can generalise the rule he has learnt. After the monkeys have reached high levels of performance on the complex tasks they will undergo operations to implant a recording chamber.

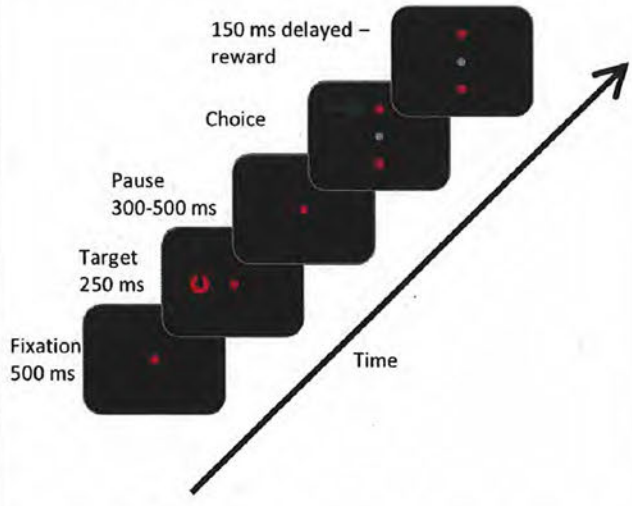

Figure 3. **Schematic overview of the task.** First, the animal has to fixate on the fixation dot in the center of the screen. Subsequently, he is presented with a peripheral stimulus to which he has read by shifting his attention without breaking fixation with the fixation dot. After a short delay, the animal has to report in which direction the gap of the peripheral stimulus was by means of a saccadic eye movement to a visual target. If the animal saccades to the right target a reward is provided.

11. Second surgical procedure: Recording chamber implantation and craniotomy (only new animal)

Discomfort: Moderate for 2-3 days, becoming mild for 1 week.

A surgical procedure is carried out under general anaesthesia to perform a craniotomy allowing access to the underlying brain structures. An incision is made in the scalp and the skin is retracted. A small section of skull (~2cm) is removed. A recording chamber is placed around the craniotomy and attached to the skull, with surgical screws and/or dental cement. Whenever possible the recording chamber will be 3D printed to ensure an excellent fit to the skull and will be made of titanium ensuring good bio-compatibility, strength and low weight. The animal receives appropriate analgesics during and after the procedure. The animal also receives drugs which reduce intracranial pressure during the operation (e.g. mannitol, dexamethasone). The duration of the procedure is approximately 1-2 hours.

The recording chamber must be cleaned every 2-3 days to prevent infection. The procedure takes place

while the animal is sitting in the primate chair before or after a recording session. The chamber lid is removed and the interior of the chamber is flushed with anti-bacterial solutions such as chlorhexidine. Finally, the chamber is flushed with saline. The total duration of the cleaning procedure is around 5 minutes and it causes no discomfort.

12. Recording sessions (all animals)

Discomfort: Mild

The neural recording sessions follow an identical format to a behavioural training session, with the exception that the monkey is connected to the recording equipment. The electrode will be carefully moved across the dura and into the brain at the start of each recording session, this causes a brief moment of mild discomfort. Subsequently, the electrode is moved into the tissue in micro-meter increments until the activity of a single cell is found. If pharmacological agents are used these will be applied via a combined recording-electrode/pipette as described I the next section. The animals will perform the same tasks as outlined above. The duration of daily sessions (max. 5 times per week) will be between 3-4 hours, including preparations. The total duration of the recording sessions will be between 2 and 2.5 years, although the chamber remains useable after this time-period.

13. Pharmacological interventions (all animals)

Discomfort: Mild

Reversible pharmacological agents will be applied locally through the implanted recording chamber, to the area of the cerebellum where task related neurons are recorded. A sharp glass pipette or combined electrode/pipette will be lowered across the dura in the same manner as a recording electrode. The pharmacological agent will either be slowly injected using pressure, or applied using iontophoresis. The approach used will depend upon the properties of the drug to be applied and the desired volume of the effect, iontophoresis produces a more local effect whereas pressure injections can affect larger volumes. For pressure injections the pipette will be connected to a small-volume syringe and a small quantity (<100nL) of drug will be slowly injected over the course of 5-10 minutes. For iontophoresis a small wire will be introduced into the glass barrel of the pipette. A holding current is applied to the wire to retain the drug within the barrel. To eject the drug, the current is switched in polarity and the charged particles of the drug are driven into the neural tissue. We will monitor the effect of the drug on neural activity through a recording electrode in an identical manner to that described above. Depending on the type of drug, suppression of activity can be up to 24 hours, therefor no more than 3 sessions per week can be performed to ensure complete washout before starting a session.

14. Removing tissue above the dura (all animals)

Discomfort: Moderate for 1 day (recovery from anaesthesia)

It is periodically necessary to remove tissue that has grown over the dura within a recording chamber to improve the ease with which electrodes can be moved into the brain. The monkey is lightly anesthetized and the tissue is removed with a specially designed tool. The amount of tissue damage caused by this procedure is minimal and the monkey recovers within an hour after cessation of the anaesthesia. The monkey receives analgesics during and after the procedure. The frequency of occurrence is approximately once per 3-6 months.

15. Third surgical procedure: Restorative surgeries (all animals)

Discomfort: Moderate for 1-2 days, becoming mild for 3-4 days.

In rare cases an implant (i.e. head-post or recording chamber) may become loose. A repair surgery is then performed to prevent failure of the implant. The repair surgery is performed under general anaesthesia with appropriate analgesia. The nature and duration of the repair surgery depends upon the type of implant and the extent of the problem, but the tissue damage imposed by the procedure is typically minimal. An individual monkey can undergo a maximum of two restorative surgeries per implant (including the head-post) during the course of these procedures. Repair surgery will always be performed in consultation with the Animal Welfare Body (IvD) and (if necessary) the veterinarian.

16. Annual health-check (all animals)

Discomfort: Mild (recovery from brief anaesthesia).

Once per year, each animal in our facility is checked by the veterinarian to assess their general health and appearance and to take blood/urine samples for further testing. In this way, the long-term health of the animals is closely monitored. The animal is lightly anesthetized during this procedure which takes 10-15 minutes per animal.

17. Transport and temporary housing at DM3 level (all animals)

Discomfort: Mild

During injections of viral vectors, it is necessary to move the monkey to a DM3 biological safety level facility due to GGO legislation. To this aim the animals will be transported to a primate DM3 facility in the Netherlands. Animals will always be transported and housed together with their cage-mate (who will also be assigned to this license) to reduce social stress. The animals will first be trained to sit quietly in the specialized transportation cage for periods of 1-2 hours by associating the transport box with positive rewards such as fruit. They will consequently not be anesthetized during the transportation and experience only mild discomfort.

18. **Injection of viral vector (all animals)**

Discomfort: Moderate for 1 day (recovery from anaesthesia)

During injections of viral vectors, it is necessary to move the monkey to a DM3 biological safety level facility. To this aim the animals will be transported to a primate DM3 facility in the Netherlands. To inject the vector, the monkey will be anesthetized. Injections will be made through the recording chamber, using an injection needle. A small gauge needle will be connected to a small-volume syringe and a small quantity (few microliters) of neuro-anatomical tracer will be slowly injected over the course of 5-10 minutes. Subsequently, the pipet will be slowly retracted to prevent spreading of the tracer to the non-target area. Since the injections are made through the recording chamber, no extra surgery is necessary. If larger volumes of vector are required, then 'convection enhanced delivery' techniques will be used: A thin cannula will be inserted into the brain and an infusion pump will be used to slowly deliver the viral vector into the brain. The animals will be housed in the DM3 facility until the viral trace has spread through the neurons, after which the animals will be euthanized and perfused.

19. Perfusion (all animals)

Discomfort: Mild or none

Due to the neuro-anatomical tracer injections animals will have to be euthanized and perfused at the end of the experiments. The animals are euthanized by an overdose of barbiturates and are then transcardially perfused with fixative.

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

The statistics in our studies are performed across neurons and behavioural trials, and we perform multiple recording sessions per animal meaning we can record a sufficient number of neurons with only two animals. Two is the absolute minimum number of animals that can be used to check for consistency across animals and is accepted as the norm in primate research. Although we have good experience with most of our monkeys, some individual circumstances may preclude a monkey from being used for a specific experiment (e.g. if he is not able to learn the behavioural task), or ambiguous results may require measurements in a third animal. In such cases, we will apply to the IvD of the institute for permission to use a third monkey.

B. The animals

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

Species used:
We will use rhesus macaque monkeys (*Macaca mulatta*) in these experiments. All monkeys are obtained from a national primate centre, or in exceptional circumstances (i.e. if no animals are available from a primate centre) from a licensed importer. Monkeys are typically acquired aged 3 years or older. The aim of this application is to understand how visuospatial attention is controlled by the cerebellum and the connections to the cortical attention related areas. This approach requires a species with comparable neuroanatomy to the human and the ability to perform attentional tasks. Rodents are able to perform some simple cognitive tasks, however there are critical differences between the functioning of the rodent and primate visual system which makes rodents unsuitable for this study. Primates have a region of their retina with extremely high spatial resolution known as the fovea, which is used for all detailed daytime vision. Primates constantly (3 times per second) make saccadic eye-movements so that the fovea of each eye are pointed at interesting regions of the visual scene. The mechanisms of visual attention are closely related to the mechanisms of eye-movement control and attention can be viewed as a 'pre-selection' of an object to plan an upcoming eye-movement. Importantly primates are able to withhold the actual eye-movement allowing them to covertly attend a particular object while keeping their eyes fixated on a different object. In rodents the control of the eyes is very different. The rodent retina is broadly speaking uniformly sensitive; they have no fovea. This means there is no need to move the eyes to fixate particular regions in the visual scene. Rodents very rarely make eye-movements and if they do these appear to be related to changes in arousal state. It appears therefore that rodents lack a mechanism for visuospatial attention making it impossible to study this process in these species. Cats have also often been used in basic visual studies, but these animals cannot be trained to perform covert attentional tasks. The experiments are invasive as they require the implantation of a head-post and further surgical implantations to allow stimulation electrodes to enter the brain. These experiments can therefore not be performed in humans. Macaque monkeys show very similar performance to humans on visual attention tasks and there is a large amount of literature on attentional processing in this species. We already have a broad outline of the anatomy of the attentional control system in macaques meaning we will be able to relate our results to previous findings making interpretation of the results much more powerful. Given these considerations, no alternative to the macaque monkey is available.

Sex used:
We exclusively use male monkeys in these studies. Our facility houses only male monkeys as males adapt better to living in paired social housing than females and there are no possible complications with breeding that would be present with female animals. The choice for males will not affect the results of the study as it is highly unlikely that there are differences between the sexes in how attention is controlled. Males are therefore chosen to allow us to maintain 100% male animals in our facility.

Animal number:
We expect that at least two animals are necessary in order to obtain reliable results for each experimental question considered. When comparable data is obtained from two individuals it can be assumed that the results are not attributable to individual differences.

The experiments described here will be used to address two sub-aims:
1) How does the cerebellum control covert attention shifts?
2) How is the lateral cerebellum connected to attention controlling areas in the cerebral cortex?

Given that to answer the second research question we can use the animals from the first after that research question is completed we will use two animals in the course of this proposal.

Previous studies have obtained reliable results from two animals per question, but given the novelty of the proposed experiments it remains hard to estimate the individual variability that we will encounter. It could be possible that data from one animal must eventually be excluded from the analysis, contradictory results arise from the first two monkeys, or ethical considerations require the termination of one animal before conclusive data is gathered. Such cases require the acquisition of data from a third animal. The

acquisition of a third animal for a particular research question will be performed in consultation with the IvD of the institute. Another group examined the use of monkeys in their lab over the past 10 years and found that in 4 out of 18 projects a third monkey was required (22% chance). Given this value it is possible that we will have to require one extra animal in addition to the two animals above. We therefore require a potential maximum of 1 newly acquired animal making a total of 3 animals.

C. Re-use

Will the animals be re-used?

☐ No, continue with question D.

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

Two animals from other previously finished experiments can be used in this proposal. They have been used in the saccade/anti-saccade experiments described in the main proposal, however in the current proposal these animals will be used for a different scientific question. In the saccade/anti-saccades experiments a similar experimental approach was used as proposed here, including single unit recordings from the cerebellum. For these experiments both animals have had recording chambers implanted, which are still in good shape and in the right spot for the experiments planned in the current proposal. The animals are already habituated to the facility and used to performing cognitively demanding visual tasks; therefore they can be rapidly employed in the proposed experiments. The cumulative discomfort of the procedures these animals have gone through is moderate.

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

☒ No

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

D. Replacement, reduction, refinement

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

Replacement

The main aim of this application is to understand how the brain controls the deployment of attention in cognitive tasks. Many studies have approached this question by using cognitive neuroscientific techniques in humans. These studies have provided valuable information about the brain areas which are involved in allocating attention and have led to a number of proposed models about the neural mechanisms of attentional deployment. Unfortunately, the temporal resolution of fMRI is not sufficient to track activity in cognitive tasks and the spatial resolution of EEG/MEG is not sufficient to localize the neural activity to particular brain regions. To fully understand the neural mechanisms that engage and shift attention we need a technique with both high temporal and spatial resolution such as invasive electrophysiological recordings. Invasive recordings can only rarely be performed in humans, typically in surgical investigations of epileptic patients who are implanted with depth electrodes to localize the source of the epileptic activity. In these studies, the location of the electrodes is based purely on clinical criteria, and they are very rarely placed in areas involved in attentional control such as the FEF or parietal cortex or cerebellum. This makes it impossible to collect sufficient neural data to gain an understanding of the underlying neural mechanisms. It is therefore not possible to replace the experiments described here with experiments in humans. It is also impossible to replace the monkeys in these experiments with rodent models or cats for the reasons outlined above in section B. Briefly, rodents and cats are unable to perform the covert attention tasks, which are essential to understanding the mechanisms of attentional deployment. Given these considerations, no alternative to the macaque monkey is available.

Reduction

The number of animals we want to use is the minimum number with which reliable results can be obtained, and no further reduction is possible. If possible, we can implant a recording chamber over the other hemisphere after recordings are no longer possible from the original sites due to damage of the