Master's thesis

NTNU
Norwegian University of Science and Technology
Kavli Institute for Systems Neuroscience

Ella Holt Holmberg

Involvement of the Anterior Cingulate Cortex during Task Learning in Rats

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Supervisor: Jonathan Whitlock
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Abbreviations

ACC	Anterior cingulate cortex
ADHD	Attention-deficit/hyperactivity disorder
ChR2	Channelrhodopsin2
Cg1	Cingulate cortex 1
Cg2	Cingulate cortex 2
CPT	Continuous performance test
DREADDs	Designer receptors exclusively activated by designer drugs
FRN	Feedback-related negativity
GN/GT	Go/no-go-task
IL	Infralimbic cortex
ITI	Inter-trial interval
LFP	Local field potential
M2	Secondary motor cortex
MCC	Midcingulate cortex
MFB	Medial forebrain bundle
mPFC	Medial prefrontal cortex
PCC	Posterior cingulate cortex
PL	Prelimbic cortex
RFP	Red fluorescent protein
SFO	Step-function opsin
SST	Stop-signal task
3-CSRTT	3-choice serial reaction time test
5-CSRTT	5-choice serial reaction time test

Abstract

The investigation of learning is an important aspect of neuroscience, as it entails a variety of processes needed for a species' survival. One brain region that has been studied in relation to learning and associated cognitive functions, is the anterior cingulate cortex. The brain region is thought to be involved in multiple cognitive functions, including attention, cost-benefit analyses, inhibition and memory. As part of a larger, ongoing PhD project investigating the involvement of anterior cingulate cortex in social learning in rats, the current project specifically investigated the involvement of the brain region in non-social, reward-driven associative learning in rats. Using optogenetic tools, we temporarily inhibited excitatory neurons in the anterior cingulate cortex while training the animals on a task consisting of ball-tapping in a specific sequence. We found that temporary inhibition of neurons in the anterior cingulate cortex did not impact the animals' ability to learn nor their accuracy on the task. It did however increase the time spent performing the task. Additionally, we found that animals trained with neurons in the anterior cingulate cortex inhibited decreased their accuracy compared to controls when tested in a later session without the active inhibition.

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1. INTRODUCTION

Learning is one of the most fundamental, yet complex features of survival. It is involved in almost every aspect of an animal's life, such as finding food, escaping predators or finding a mate. Simply put, learning can be defined as how an organism integrates information from its environment and adapts its behavior accordingly. It encompasses a wide array of processes and behaviors, such as the habituation of a single-celled organism to stimuli (Boisseau et al., 2016), synaptic plasticity needed for memory formation (Bliss & Lømo, 1973), and the complex neural processes needed for creative problem solving (Dietrich & Kanso, 2010). Investigating learning and its principals is therefore a cornerstone in the field of neuroscience.

1.1 Classical Learning Principles and Cognitive Tasks

Much of the research on learning today still relies on the classical learning principles introduced by Ivan Pavlov and Burrhus Frederic Skinner. In brief, these learning principles state that 1) an arbitrary stimulus can become a conditioned stimulus through associations and 2) an organism will either reduce or increase a behavior depending on whether the behavior is rewarded or punished (Holt et al., 2015, p. 272-281). The former learning principle is termed classical conditioning and refers to an organism's tendency to associate unrelated stimuli if they appear in close proximity to each other. For example, if a neutral unconditioned stimulus in the form of a tone is played just before a mouse receives a foot shock, the tone will quickly be associated with the foot shock and can by itself induce a fear response in the mouse at a later time (Kandel et al., 2013, p. 1084). The tone has then become a conditioned stimulus and the fear response has become a conditioned response. The latter learning principle is termed operant conditioning and refers to the association between a voluntary behavior and its consequences. If a mouse experiences that pressing a lever results in obtaining a reward, the action will be reinforced, and the mouse will quickly learn to press the lever in order to get the reward (Iversen, 1992). If subsequent lever presses no longer result in obtaining a reward, the mouse will eventually stop performing the previously rewarded action, a process termed extinction learning (Bouton, 2004). If the lever press instead results in a foot shock, the animal quickly learns to not press the lever (Bouton, 2015). The learning principles outlined here are common across most tasks used to study the different aspects of learning.

1.1.1 Rodent Paradigms Assessing Learning and Related Cognitive Functions

There exists a large range of tests and paradigms used to measure learning and related cognitive functions, many of which can be applied across species. Despite anatomical and functional differences between the rodent and the human brain, rodent models have been widely used to study cognitive functions. The use of rodents in cognitive research gives the valuable opportunity of using intracranial neural recordings and targeted manipulations of neural circuits, in combination with behavioral and cognitive assessments. Combined with the fact that rodents, like humans, displays higher cognitive functions needed for goal-directed behavior, such as planning, decision-making and behavioral control, it makes rodents valuable as a model to study cognitive functions (see Chudasama, 2011, for a review on the use of rodents to investigate cognitive-executive functions). Following is a brief introduction to some of the tests commonly used with rodent models. While several of these paradigms are modified in individual experiments, the main components remain the same.

Some paradigms take advantage of the basic Pavlovian principles to assess learning in different forms. During **fear conditioning**, an animal learns that certain stimuli predict aversive events (Maren, 2001). The previously mentioned pairing of a tone and a foot shock has been shown to reliably lead to defensive behaviors such as freezing when presented with the tone alone at a later time (Kandel et al., 2013, p. 1084). By testing the animal's reaction to a conditioned stimulus after a prolonged period (days, weeks or months), this paradigm can be used to assess memory and long-term stability of a learnt association.

In Pavlovian **autoshaping** paradigms, one takes advantage of an animal's tendency to physically approach a stimulus that has been associated with positive reinforcement, as first described by Brown & Jenkins (1968). This approach occurs even though the approach itself has no influence on the delivery of the reward, and this way the animal's associative learning can be assessed. For instance, if an animal is presented with a variety of visual stimuli, but only some are followed by the delivery of a reward, the animal's ability to correctly associate certain stimuli with reward can be measured as the proportion of approaches to the rewarded stimuli over the unrewarded stimuli (e. g. Bussey et al., 1997a).

Similarly, **novelty preference** takes advantage of some animals' tendency to spend more time investigating novel compared to familiar visual stimuli or objects and is a popular method to assess memory and visual discrimination both in human infants and animals (e. g. Ennaceur & Delacour, 1988; Slater et al., 1983; Snyder et al., 2008). By measuring time

spent looking at or investigating familiar versus unfamiliar objects or visual stimuli, memory for the familiar object or visual stimuli is assessed.

Visual discrimination tasks are different cognitive tasks with the common denominator that the subject needs to discriminate between different visual stimuli. A simple type of visual discrimination task is a 2-choice task in which the subject needs to learn a rule such as "if stimulus A, press left lever, if stimulus B, press right lever" in order to successfully obtain a reward (Bussey et al., 1996). In concurrent visual discrimination tasks, the subject needs to learn which stimulus, out of more than one, with which interaction results in obtaining a reward, for example having rats press their snouts to the correct stimulus on a touchscreen filled with other non-target stimuli. This is sometimes done with single pairs of stimuli but can also be extended to multiple single stimuli or multiple pairs of stimuli in order to increase the cognitive load (e. g. Bussey et al., 1997b). The performance on such tasks is seen as a measure of learning, memory and attention (Steckler & Muir, 1996).

A popular visual discrimination task used both in humans and animals is the **5-choice serial reaction time test** (5-CSRTT) or a modified version with only 3 choices (3-CSRTT). In the rodent version, brief light flashes are presented in one of five holes on a panel in an operant chamber. The animal must respond to the corresponding spatial location, for example with a nose poke into the hole marked by the flashes of light, to obtain a reward. As there is a limited time window for the animal to respond, the task can measure aspects like reaction time, accuracy and premature and perseverative responses. In some versions, distracting stimuli is added to increase the cognitive load (e. g. Fisher et al. 2020). The 5-CSRTT and 3-CSRTT are seen as measures of sustained and spatial attention (e. g. Chudasama et al., 2003; Wu et al., 2017) and inhibition and impulse control (e. g. Bari et al., 2008).

A similar paradigm to assess sustained attention, but also inhibition, are **continuous performance tests** (CPT). There are many varieties of these tests, but what they have in common is the requirement of actively responding to relevant stimuli while suppressing a response to non-target stimuli. The two most popular versions of CPT are the go/no-go-task (GN/GT) and stop-signal task (SST) (McKenna et al., 2013). In both tasks, the animal must first learn a rule. An example is a simple reaction time task where the animal must press a lever when presented with a specific visual stimulus on a screen to obtain a reward. On some trials, a stop-signal, for instance a tone, is presented. The stop-signal is presented infrequently

and signals that the animal must inhibit its now habitual response in order to obtain a reward (Purves et al., 2013, p. 442). The difference between the GN/GT and the SST is the timing of the stop-signal. In GN/GT, the stop-signal is presented before the go-signal and in SST the stop-signal comes after the go-response is initiated, thereby requiring slightly different inhibitory behaviors and neural mechanisms (Raud et al., 2020). The animal's ability to inhibit its habitual response is seen as a measure of inhibition and attention. These CPT's can be combined with other tasks. For example, the 5-CSRTT can be combined with a GN/GT, called a 5-choice CPT. Here, a stop-signal is added to the previously described 5-CSRTT and is hence a better measure of vigilance than the original task alone (Young et al., 2009).

1.2 The Anterior Cingulate Cortex

To start unraveling the complex nature of learning it can be useful to study the involvement of individual brain regions. One such brain region which has been studied in relation to cognitive functions involved in learning in both primates and non-primate mammals is the anterior cingulate cortex (ACC). The ACC is a functionally diverse, fronto-cortical brain region, which faces towards the midline of the brain and surrounds the corpus callosum. It consists of multiple highly inter- and intra-connected subdivisions, with projections to regions such as the basolateral amygdala, hippocampus, ventral striatum, ventral tegmental area, and sensory and associative cortices (see Burgos-Robles et al., 2019 for an overview of connectivity). The ACC is thought to be involved in a wide array of higher cognitive functions, such as attention (e. g. Alexander et al., 2005; Weissman et al., 2005), error-detection (e. g. Emeric et al., 2008; Godlove et al., 2011), reward evaluation (e. g. Monosov, 2017; Umemoto et al., 2017) and processing of social information (e. g. Apps et al., 2016; Behrens et al., 2008; Chang et al., 2013; Schneider et al., 2020), to name a few. The focus of this thesis is the rodent ACC and its role in learning. Though the rodent and primate ACC are anatomically distinct, many of the suggested functions of the ACC are found to be conserved across species (Burgos-Robles et al., 2019; Paxinos & Watson, 2014; Vogt & Paxinos, 2014). This similarity makes the rodent model useful to investigate ACC's functions with potentially translational results.

1.2.1 Anterior Cingulate Cortex Delineation

Across the literature on the ACC, inconsistencies in the definitions and nomenclature used to describe and delineate the ACC can be found. In rodent research, the view that the

ACC constitutes the cingulate cortex 1 & 2 has been popular (Heukelum et al., 2020). However, some definitions of the ACC also include the infralimbic cortex (IL) and prelimbic cortex (PL), in addition to the cingulate cortex 1 & 2 (Cg 1 & 2) (Vogt & Gabriel, 1993). This definition of the ACC has been used interchangeably with the medial prefrontal cortex (mPFC) by some (see Laubach et al., 2018 for a discussion of inconsistencies in nomenclature). In their 2014 rodent brain atlas, Paxinos and Watson revised their definition of the ACC using Brodmann nomenclature in order to bridge the research on rodents and primates (fig. 1.1) (Paxinos & Watson, 2014). This definition includes area 24a (Cg2), 24b (Cg1), 32 (PL), 25 (IL) and area 33. Area 33 is located ventral to 24a (Cg2) and is only present in rats, not mice (Vogt & Paxinos, 2014). In recent years, some researchers have chosen to separate area 24 into area 24 and 24'. The former has been considered a part of the ACC and the latter has been termed the midcingulate cortex (MCC) (see van Heukelum et al., 2020 for a review and Vogt & Paxinos, 2014 for a justification of the inclusion of MCC).

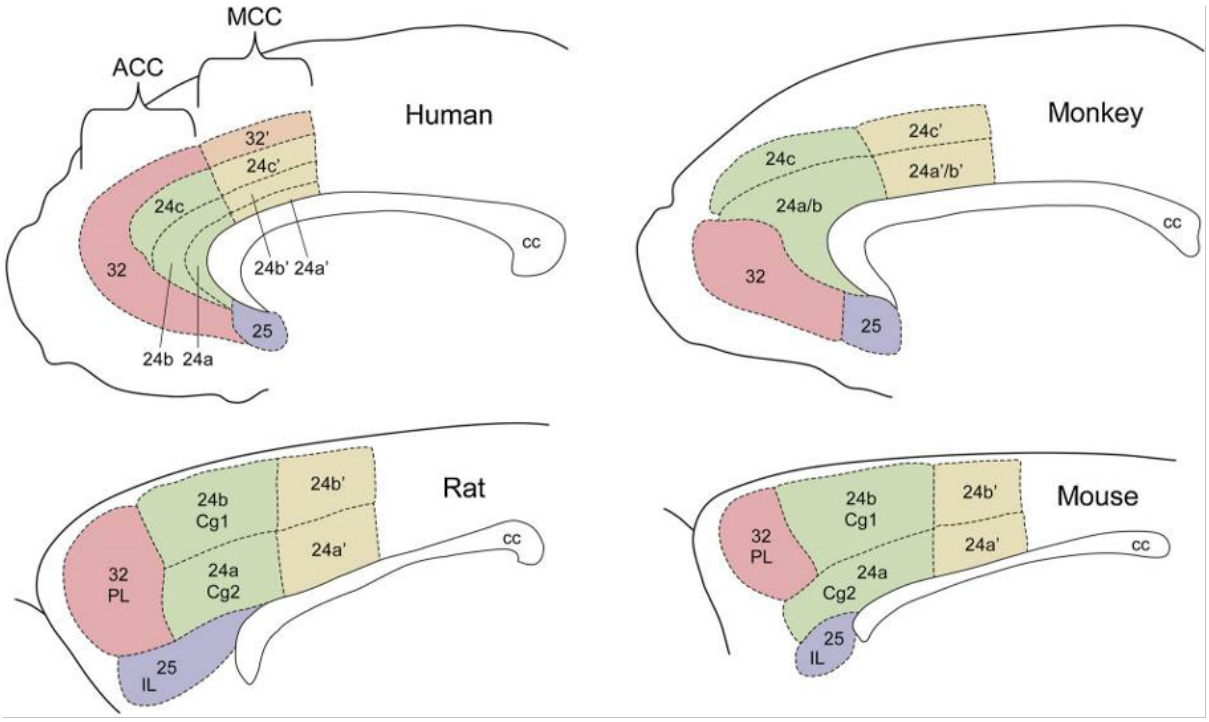

Figure 1.1 Midsagittal sections from primates and rodents, showing the brain regions constituting the ACC. The alphabetical labels show the definition of the ACC which includes the prelimbic cortex (PL), infralimbic cortex (IL), and cingulate cortex 1 & 2 (Cg 1&2). The numbers represent the Paxinos & Watson 2014 use of Brodmann nomenclature. Additionally, the human section shows the division of the anterior cingulate cortex (ACC) and the midcingulate cortex (MCC). Figure adapted from Burgos-Robles et al. (2019). This figure does not include area 33.

Given the inconsistencies in nomenclature and delineation, research on the ACC must be read with some caution. When reviewing the literature on the field, I have included

research operating within the wider, but accepted, definition from the Rat Brain by Paxinos & Watson from 2014, also in cases where the authors use the term mPFC instead of ACC.

1.3 ACC's Role in Learning

A substantial amount of research has been devoted to the role of the ACC in the acquisition of task rules and performance in a variety of the tasks outlined previously. As a result, some of the suggested functions of the ACC includes stimulus-reinforcer associations (e. g. Bussey et al., 1997b, Cardinal et al., 2003), attention (e. g. Fisher et al., 2020; Kim et al., 2016; Totah et al., 2009), inhibition (e. g. Bryden et al., 2019; Hvoslef-Eide el al., 2018; Narayanan et al., 2006), error-detection (e. g. Hyman et al., 2013; Warren et al., 2015), reward estimation (e. g. Schweimer & Hauber, 2005; Walton et al., 2003) and long-term memory (e. g. Restivo et al., 2009; Vetere et al., 2011). At first glance, it can seem as if the ACC plays a crucial role in a broad spectrum of cognitive functions. It is therefore important to note that many of these suggested functions are overlapping. For example, attention is likely needed to detect and process a stop-signal which in turn would lead to inhibition. Moreover, one would assume that the neural coding of reward expectations and long-term memory would be needed for error detection. In their review, Laubach and colleagues (2015) chose the term "adaptive control" to describe the function of the ACC, which can arguably be an encompassing term for many of the different suggested functions. The most prominent findings regarding ACC functions will be discussed below, ranging from older studies directly focusing on learning itself, to more recent research investigating more specific but related cognitive aspects assumed to underlie the process of learning.

1.3.1 Stimulus-Reinforcer Associations and Early Learning

In the 1990s, Bussey and colleagues did multiple quinolinic acid induced lesion studies on the ACC and the posterior cingulate cortex (PCC) in rats to probe their ability to learn different conditioned visual discrimination tasks through reinforcement (Bussey et al., 1996; Bussey et al., 1997a; Bussey et al., 1997b). Rats with lesions in the ACC showed significantly poorer discrimination learning in Pavlovian autoshaping experiments when compared to unlesioned animals (Bussey et al., 1997a; Bussey et al., 1997b, Cardinal et al., 2003). ACC lesions have additionally been found to impair the performance on pre-trained autoshaping tasks compared to performance before lesioning (Cardinal et al., 2002). It also led to significantly poorer performance on an eight-pair visual discrimination task, a task in

which the rats had to learn which stimulus, out of eight pairs of stimuli, must be pressed on a touchscreen in order to obtain a reward (Bussey et al., 1997b).

Some of this research has indicated that the ACC might be needed for the stimulus-reinforcement associations required during the early stages of learning, but not for the late-learning phases which involve developing habitual stimulus-response associations. On autoshaping tasks, ACC lesioned rats eventually learned to discriminate between visual stimuli but required more sessions and never performed at the same level as unlesioned controls (Parkinson et al., 2000). Bussey et al. (1996) showed that in the first three sessions of an extinction task, ACC lesioned rats persistently continued pressing a lever in the absence of reward while unlesioned controls quickly reduced their lever pressing when no reinforcer was given. The lesioned animals did, however, show extinction of the behavior in subsequent sessions. Gabriel et al. (1990; 1991) performed multiple experiments on avoidance learning in rabbits, in which one tone (conditioned stimulus) preceded a foot shock (unconditioned stimulus) and another tone did not have an outcome and was to be ignored. The rabbit could avoid the foot shocks through stepping on a running wheel after hearing the conditioned tone. Multi-unit recordings from the ACC showed increased neuronal activity following the presentation of the tone preceding the foot shock compared to the irrelevant tone, especially during the initial trials (Gabriel, 1990). When the rabbit gradually learned to avoid the foot shocks through stepping on the running wheel, the neuronal response to the conditioned stimulus decreased, in accordance with the idea that ACC is mainly involved in early phases of learning when stimulus-reinforcer associations are developed.

In contrast, Bussey and colleagues saw facilitation of early learning in ACC lesioned rats in a single-pair visual discrimination task (Bussey et al., 1996). In this specific experiment, the rats had learned to associate a lever press with a reward prior to lesioning and needed only to learn a single stimulus-response rule after lesioning (Bussey et al., 1996). This discrepancy from the impaired learning seen in their previous experiments led the authors to speculate that the neural circuits for early- and late learning are complementary but competing systems, and that the former would utilize the ACC and the latter the PCC. A compromised stimulus-reinforcer system, the ACC, could lead to less competition for the stimulus-response system, thus facilitating the learning process when the stimulus-reinforcers associations were already formed.

As shown, there has long been evidence for a role of the ACC in the development of stimulus-reinforcer associations necessary during the initial phases of learning. However, these early studies do not go into detail on the specific cognitive processes involved in learning. As the following paragraphs will describe, the more recent literature has tended to switch the focus from investigating learning as a general concept to instead focus on different underlying cognitive functions that might be necessary for learning.

1.3.2 Attention

One of the prominent hypotheses for ACC function is its involvement in control of attention, which is typically assessed using tasks like the 3-CSRTT, the 5-CSRTT, simple reaction tasks and rodent CPT's. These tasks include measures such as accuracy and response time in face of distracting stimuli and can thus be seen as a measure of attention, as the animal needs selective focus in order to perform the tasks accurately and fast. Rodent studies using these tasks have found impaired accuracy (Chudasama et al., 2003; Fisher et al., 2020; Passetti et al., 2002; Wu et al., 2017), increased premature responses (Hvoslef-Eide et al., 2018; Muir et al., 1996; Narayanan et al., 2006; Wu et al., 2017), increased response latencies (Passetti et al., 2002) and increased omissions (Chudasama et al., 2003; Passetti et al., 2002) following ACC lesions.

Using a Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based tool, pan-neuronal inactivation of the dorsal ACC led to increased omissions and longer correct response latencies on a 5-CSRTT (Koike et al., 2016). When the researchers instead specifically inhibited excitatory neurons, they saw decreased accuracy in addition to the increased omission, but not increased latencies for correct responses. A within-subject experiment found that temporarily inhibiting neurons in the ACC through infusions of the GABA-A receptor agonist muscimol resulted in impaired accuracy on a visual discrimination task only in the presence of task-irrelevant, distracting stimuli, indicating a lack of selective attention (Kim et al., 2016). In support of the ACC having a role in attention, recordings in the region have revealed neurons firing before the presentation of a cue to which the animal should be attentive (Totah et al., 2009) as well as during periods where sustained attention was required to complete a task (Wu et al., 2017).

1.3.3 Inhibition, Error-Detection and Cost-Benefit Analysis

Partly overlapping with attention, some research has suggested that the ACC is needed for inhibition. To study inhibition, it is common to use tasks where a pre-trained response is supposed to be inhibited, like the previously mentioned different CPTs. Muscimol infusions in the ACC of rats have been found to increase response time and impair stop accuracy on an SST (Bari et al., 2011). Concordantly, single-unit recordings from the ACC in rats during a stop-change task revealed increased firing rates during stop-trials, especially when the trial followed a go-trial (Bryden et al., 2019). The ACC's involvement with inhibitory functions is further supported by the previously mentioned premature responses seen after lesions (Hvoslef-Eide el al., 2018; Narayanan et al., 2006; Wu et al., 2017). Associated to a lack of inhibition, Chudasama et al. (2003) found that lesioning the ACC in rats led to an increase in compulsive or perseverative behavior when the inter-trial intervals (ITI's) were short, but not with longer ITI's. The perseverative behavior was measured as additional responses to the correct location in a 5-CSRTT following a correct response. One possible explanation for the disinhibited behavior could be attributed to increased hyperactivity in rats with impaired ACC functioning. While indeed some literature indicates that ACC lesions leads to hyperactivity measured as increased locomotion (Rudebeck et al., 2006), many studies report no change in locomotion between lesioned groups and controls (Cardinal et al., 2002; Koike et al., 2016; Narayanan et al., 2006; Wu et al., 2017), which leaves little evidence in favor of the hyperactivity hypothesis. Thus, the reduced inhibition seen following ACC lesions is presumably not a result of mere hyperactivity.

Other studies have investigated error-detection in the ACC. Narayanan and Laubach (2008) found a decrease in post-error slowing, the extra time spent on a subsequent trial after an erroneous trial, on a simple reaction time task after lesioning the ACC of rats. When doing single-unit recordings during the same task, the researchers found a subpopulation of neurons which increased their firing persistently in response to performed errors, and this firing accompanied the post-error slowing. Similarly, Hyman et al. (2013) recorded a subpopulation of neurons in the rat ACC that fired in response to the outcome of a trial, with the majority of neurons firing in response to erroneous trials. In human EEG studies, researchers have seen a negative neural deflection signal in response to feedback indicating an incorrect performance, termed feedback-related negativity (FRN), as first described by Miltner et al. (1997). The FRN was suspected to stem from the ACC, but no conclusions were drawn given the spatial imprecision of EGG data (Miltner et al., 1997). Using local field potential (LFP) recordings

from the ACC, Warren et al. (2015) found neural activity resembling FRN in rats. Nose pokes in three different ports elicited a reward with varying probabilities, and two different scents were used to indicate whether or not a reward would be given. Measuring the differential response between a positive and a negative outcome, the researchers saw an FRN-like negative neural deflection following a scent indicating that the reward would not be given (Warren et al., 2015).

Lesions to the ACC in rats can additionally influence their reward evaluation and cost-benefit decisions. Using a T-maze, studies have found that unlesioned rats chose to climb a barrier to obtain a larger reward instead of accepting an easier to reach but smaller reward, while ACC lesioned rats preferred the low-cost but low-reward option (Rudebeck et al., 2006; Schweimer & Hauber, 2005; Walton et al., 2003). If there was a barrier to both the low and high reward, the lesioned rats reliably chose the higher reward, which implies that the rats were still able to accurately infer the difference in reward size. Interestingly, the ACC lesioned rats still preferred to wait 15 seconds for a higher reward instead of an immediate but lower reward (Rudebeck et al., 2006). These results indicate that while lesions to the ACC does not disrupt the ability to assess the size of a reward, it can alter the cost-benefit analysis. Given the previously described disinhibitory behaviors following ACC lesions, it can be speculated whether these altered cost-benefit decisions are due to disinhibition rather than a deficit in reward evaluation. This would coincide with what is often seen in attention-deficit/hyperactivity disorder (ADHD) in humans, where a lack of inhibition can lead to sub-optimal decision making (e. g. Groen et al., 2013; Marx et al., 2018). On the other hand, if the lack of inhibition was the only explanation for these findings, one would expect the ACC lesions to reduce the rats' willingness to wait longer for a higher reward, contrary to what was reported by Rudebeck et al. (2006).

1.3.4 Long-Term Memory

In addition to the previously mentioned potential functions of the ACC, there is evidence for a role in the formation of long-term memories in the ACC through its connectivity with the hippocampus. In mice, an increase in spine density, an indication of the synaptic plasticity needed for long-term potentiation, could be observed in the hippocampus 48 hours after fear conditioning (Restivo et al., 2009). Long term (36 days), this increase in spine density was be found in the ACC instead. Restivo et al. (2009) also found that lesioning the hippocampus immediately after the fear conditioning disrupted spine growth in the ACC

and the associated memory recall, measured as freezing behavior when the mouse was placed back in the conditioning chamber. No such change in memory recall was seen if the hippocampus lesion was performed 24 days after the fear conditioning. These findings are in accordance with the theory stating that the hippocampus has a time-limited role in driving structural changes needed for long-term memory consolidation (Frankland & Bontempi, 2005). Concordantly, transient inactivation of the ACC disrupts recall of remote but not recent fear memories (Frankland et al., 2004), and inhibition of spine growth in the ACC one day after fear conditioning, but not 42 days after, disrupts later memory recall (Vetere et al., 2011).

Extending outside the fear conditioning paradigm, long-term memory in the ACC has also been tested with spontaneous object recognition (Pezze et al., 2017; Weible et al., 2012). Muscimol infusions in the rat ACC led to similar exploration time spent on a familiar object versus a novel object when infused before a 24-hour retrieval phase, indicating a lack of memory for the familiar object. When muscimol was infused before the encoding phase, or before a retrieval phase 20 minutes after encoding, the rats did not differ from controls on time spent investigating the familiar object (Pezze et al., 2017). This further supports the idea that the ACC is involved in long-term, but not short-term, memory. Concordantly, Weible et al. (2012) recorded neurons in the ACC in mice that responded to the absence of an object in a familiar environment. 6 hours after exploring two objects in an arena, one object was removed, and the mice were placed back in the arena. Some ACC neurons continued to respond to the location where the object had been, indicating a memory for the removed object and its location.

1.3.5 ACC Summary

As described above, the ACC is a functionally diverse brain region, involved in a range of cognitive functions. The highlighted cognitive functions can presumably impact an animal's ability to learn under certain conditions. For example, error-detection is needed to continuously update and improve a learning strategy, reward evaluation and cost-benefit decisions can influence the motivation to perform and hence develop a learned response, and attention can be necessary in order to gain information about the surroundings and increase memory function. It is difficult to pinpoint the exact contribution of these separate functions to learning, in addition to determine how separate these functions are. In the experiments where the ACC was compromised, either through lesions, DREADDs, or muscimol

infusions, the animals were typically able to perform or learn a variety of tasks, although usually not as well or as fast as control animals with a fully functioning ACC. The exact contribution of the ACC to learning in rodents seems thus yet to be determined.

1.4 The ACC and Observational Learning

In addition to the aforementioned functions of the ACC, there is evidence that the ACC might also contribute to a type of social learning termed observational learning. Observational learning refers to the ability to change or adapt one's own behavior based on information received through observing someone else performing an action, usually a conspecific. Observational learning has been extensively studied and shown across a range of species, ranging from fish to birds to apes (e. g. Carlier & Jamon, 2006; Whiten et al., 2005; see Bennett & Laland, 2005 for a review). It can for example provide an animal with crucial information on how to access food, which foods are safe to eat and how to avoid predators without themselves being at risk of harm.

Previous research has found a crucial role for the rodent ACC in observational fear learning, meaning the social transfer of fear from one animal to another through observation (see Kim et al., 2018 and Keum & Shin, 2019 for reviews). The observational fear learning paradigm usually consists of mice witnessing other mice receiving foot shocks (Allsop et al., 2018; Carillo et al., 2016; Han et al., 2019; Jeon et al., 2010; Keum et al., 2018; Kim et al., 2012). Afterwards, the fear response of the observer mouse is quantified. Inhibition of neurons in the ACC disrupts the acquisition of fear during observational learning, seen as an absence of or reduced freezing response when placed back in the environment in which the fear conditioning took place, compared to mice without neurons in the ACC inhibited.

In contrast, inhibition of neurons in the ACC does not affect the fear response after mice experience the foot shocks first-hand. Neither does it appear to affect memory retrieval of already acquired fear when tested 24 hours after fear conditioning (Allsop et al., 2018; Jeon et al., 2010). These results might seem inconsistent with the previously mentioned studies on ACC's involvement in long-term memories. However, there might be differences in memory consolidation for emotionally arousal stimuli like foot shocks compared to less arousal stimuli, like the memory of an object. Moreover, while the studies on object recognition found evidence of long-term memories in the ACC 24 hours after encoding, the studies on memories for fear conditioning indicated that the memory formation for fear happened on a longer time scale.

These findings on ACC's involvement in observational fear learning has led to the question of whether the role of ACC also generalizes to social learning outside the fear paradigm. Neurons in the ACC have been found to respond to both aversive and rewarding stimuli in oneself and when observing a conspecific receive the same stimuli (Schneider et al., 2020), but there is a lack of research focusing specifically on observational, reward-driven learning. The PhD project of which this thesis is a part of aims to fill this gap in the literature.

1.5 The Observational Learning Paradigm and the Current Study

In the PhD project developed by my co-supervisor, Ida V. Rautio, a naïve rat witnesses a pre-trained rat performing a task consisting of tapping two ping-pong balls in a specific sequence in order to obtain a reward. For every successful trial performed by the performer rat, both the performer and the observer rat obtain a reward. After observing the task for 30 minutes on three consecutive days, the observer rat is tested on the same task in order to ascertain whether observational learning has occurred. In one condition, neurons in the ACC of the observer rat are optogenetically inhibited during the observation phase, to test for the contribution of the ACC during learning through observation.

The current experiment was designed to control for the ACC's contribution to non-social learning, without having the rat observing a conspecific performing the task. This was done in order to single out the observational aspect in the observational learning experiment. To this end, we manually trained naïve rats to perform the ball-tapping task, in which they had to learn a non-intuitive tapping sequence in order to obtain a reward. The animals were rewarded at intermittent steps, also known as shaping, until the whole sequence was performed before a reward was given to the animal. The rats were divided into two groups, one control group without any manipulation of neurons in the ACC and one experimental group with rats with excitatory neurons in the ACC temporarily inhibited during the training sessions.

To reward the rats, a bipolar electrode implant targeting the medial forebrain bundle (MFB) was used. The MFB is a neural pathway involved in the reward system, and stimulation of this pathway is associated with reward and pleasure (Hernandez et al., 2006). Intracranial stimulation was chosen as it is an immediate and reliable way of delivering a reward. This is necessary for training through shaping as the temporal proximity of a reward

to the correct behavior is important creating the correct associations. Additionally, it removes the need for food- or water deprivation often used in paradigms relying on rewards.

To temporarily inhibit excitatory neurons in the ACC, we used optogenetic tools. Specifically, we injected into the target area a viral vector transporting Channelrhodopsin2 (ChR2), a light-gated cation channel, with a peak response to light at 470 nm (Lin, 2009; see Yizhar et al., 2011a for review on optogenetic tools). The rats were then implanted with an etched bilateral fiber-optic cannula implant targeting the center of the viral injections in the ACC, approximately on the border between cingulate cortex 1 & 2. Through targeting GABAergic interneurons with a pan-interneuronal enhancer (mDlx), we excited the interneurons in the target area with light stimulations, which resulted in inhibition of surrounding excitatory neurons (Dimischstein et al., 2016).

Overall, the aim of the current study was to investigate how temporary inhibition of neurons in the ACC in rats would impact reward-driven non-social learning. As described, previous studies have found that inhibition or lesions of the ACC in rodents do not diminish learning per se, but it can to some degree disrupt some of the cognitive functions related to learning. Previous studies found that ACC lesioned animals could learn a variety of tasks but needed more sessions than control animals (Bussey et al., 1997a; Bussey et al., 1997b; Gabriel 1990; Parkinson et al., 2000). Additionally, studies using attentional tasks have found reduced accuracy (Chudasama et al., 2003; Fisher et al., 2020; Passetti et al., 2002; Wu et al., 2017), longer correct response latencies (Koike et al., 2016; Passetti et al., 2002) and increased perseverative behavior under certain conditions (Chudasama et al. 2003) on different cognitive tasks following ACC inhibition or lesions.

We hypothesized that the rats with temporary inhibition of excitatory neurons in the ACC would 1) increase the number of sessions needed to learn the task, 2) show a reduced accuracy on the task compared to control animals and 3) show increased response latency on the task compared to control animals. Additionally, while the data on perseveration is ambiguous, we wanted to explore whether ACC inhibition would lead to more perseverative behavior, seen as perseverative tapping of the ping-pong ball where they last obtained a reward.

2. METHODS

2.1 Animal Housing and Husbandry

The project was approved by the Norwegian Animal Research Authority and was conducted in accordance with the Norwegian Animal Welfare Act §§ 1-28 and the local directives of the responsible veterinarian at the Norwegian University of Science and Technology. All rat handling, housing, breeding and experimental procedures for this thesis was carried out in the animal research facility at Kavli Institute for Systems Neuroscience.

A total of ten 13-19 week old male Long Evans rats weighing 400-500 grams at the time of surgery, bred in-house, were used in the experiment. The animals had access to food and water ad libitum and were kept at a reversed 12-hour light/dark cycle. Room temperatures were kept between 20 and 24 degrees C and the air humidity between 40 and 70%. The animals were housed together with same-sex litter mates in enriched cages until implantation. One or two days before implantation, the rats were moved to a separate cage to get familiarized with single housing, which was done to avoid potential complications following implantation.

2.2 Habituation and Handling

2.2.1 First Stage of Habituation

The rats were regularly handled from 6-9 weeks of age. In addition to receiving treats, tickling was used to relieve stress and create positive associations to human handling (LaFollette et al., 2017). When comfortable with basic human handling, the rats were then brought out of the cage, both together with their littermates and individually in sessions of minimum 10 minutes and up to 1 hour. Rats that expressed high levels of anxiousness in response to handling after multiple habituation sessions were excluded from the study at this stage.

2.2.2 Habituation to the Experimental Set-Up

When the animals had reached a minimum of 11 weeks of age and were sufficiently habituated to human handling, the animals were habituated to the experimental set-up. After showing signs of relaxation, like eating and grooming on the researcher's lap, the rat was

placed inside the observer chamber of the experimental box (fig. 2.1) with treats for 30 minutes. This was repeated once a day until the rat was deemed well habituated to the experimental set-up, for a minimum of two sessions but no specific maximum limit. Signs that the animal was well habituated included a minimal number of feces, the animal eating the treats and grooming themselves, and in some cases, the rat falling asleep while being in the experimental box. After implantation, the rat would be given two additional 30 minutes long habituation sessions in the performer chamber of the experiment setup, untethered the first session and habituated to being tethered the second session. These sessions were without treats.

2.3 Surgeries

Two different surgical protocols were followed depending on whether the rat was to be used in the experimental or the control group. All surgical interventions were done in accordance with the ethical guidelines. The animals used in the control group were implanted with a single bipolar electrode targeting the medial forebrain bundle (MFB). The animals in the experimental group underwent a bilateral viral injection targeting the border between cingulate cortex 1 (Cg1) and cingulate cortex 2 (Cg2), a unilateral electrode implantation targeting the MFB and a bilateral fiber-optic cannula implantation targeting the border between Cg1 and Cg2. The fiber-optic cannula implant and the electrode implant were implanted concurrently. Devika Kurup implanted two animals and Ida V. Rautio injected two and implanted five of the animals used for this thesis, while the remaining surgical procedures were performed by the author.

2.3.1 General Surgical Procedure

The surgery room and tools were prepared and sterilized prior to surgery. In addition to cleaning the surgery room with soap and 70 % ethanol (VWR International, Ltd., USA), the surgery room was sterilized every night with radiating UV-light. Necessary tools were lined up on a surgical drape (OP-towel, Barrier Healthcare Ltd., UK). Prior to surgery, the rat was weighed, and analgesic and anesthetic agents were prepared according to their weight. All surgical procedures were performed on a down-ventilated surgery table.

The rat was anesthetized with 5% isoflurane (Isoflo vet 100%, Zoetis Inc., USA) in an induction chamber and then placed on a towel on a heating plate. Surgical anesthesia was maintained with an isoflurane concentration at between 1.5 and 3 % and with a flowrate of

0.3-0.4 l/M. In addition, the rat received oxygen at a flowrate of 0.3-0.4 l/M. The fur on the rat's scalp was removed, and the claws were cut to avoid scratching after surgery. Metacam (2 mg/ml, meloxicam, Boehringer Ingelheim GmbH, Germany) and Temgesic (0.03 mg/ml, Schering-Plough, USA) were administered subcutaneously. Marcain (2.5 mg/ml, bupivacaine, AstraZeneca AB, UK) was injected subcutaneously on the scalp. In addition, the rat was injected subcutaneously with 12.5 mL of saline (9 mg/ml, B. Braun Melsungen AG, Germany) to avoid dehydration, with half the dosage injected at the beginning and the other half towards the end of the surgery. The rat's eyes were covered with Simplex (Optha A/S, Denmark) to prevent drying.

The rat's head was tightly fixed to the stereotaxic frame (Kopf Instruments, USA) with ear bars. Chlorhexidine (5 mg/ml, Fresenius SE & Co. KGaA, Germany) was applied with cotton swabs to the shaved area of the rat's head for disinfection. When the local anesthetic injected on the scalp had diffused into the tissue, a straight midline incision was made with a surgical scalpel (size 22, Swann-Morton Ltd., UK), and the skull exposed. Four hemostatic clamps were attached to underlying tissue and taped to the stereotaxic frame in order to expose the skull. Hydrogen peroxide (NAF 3%, Norges Apotekerforening, Norway) was used for disinfection, and excess and damaged tissue was removed using microscissors as to lower the risk of post-surgical infections.

2.3.2 Viral Injections

The viral vector used (AAV5-mDIx-Chr2-mCherry-Fishell-3) was prepared at the Viral Vector Core Facility at the Kavli Institute for Systems Neuroscience by Rajeevkumar Raveendran Nair. The promoter mDlx specifically targets GABAergic interneurons. Light stimulation of interneurons expressing the light-gated cation channel Channelrhodopsin2 excites the interneurons which in turn inhibit surrounding excitatory neurons (Dimidschstein et al., 2016). The viral vector was stored at -80 degrees C. Prior to surgery, a small concentration of Fast Green (Fast Green FCF, Merck KGaA, Germany) was added to the viral vector to allow for visual confirmation of injection during the procedure and the viral vector was inserted in a pulled glass microcapillary pipette (World Precision Instruments Inc., USA).

The viral injections were performed when the rats were 9-10 weeks old (N=4) to allow enough time for viral expression before the experiment. A craniotomy was made with a 1 mm burr (Fine Science Tools, USA), the dura was punctured, and the viral vector was

injected at the following coordinates from bregma: AP: + 2,5 ML: ±0,5. The injection was initiated while slowly lowering the pulled glass microcapillary pipette containing the viral vector into the brain, using a microinjector pump (World Precision Instruments Inc, USA). A bilateral injection of 500-700 nL was performed at the same coordinates as the craniotomy, with an end depth of DV: 2 measured from dura, targeting the border between cingulate cortex 1 & 2. The rate of injection was 50 nL/min. After injection, the microcapillary pipette was kept in place for 10 minutes to avoid backflow. The process was repeated in the other hemisphere, using the same microcapillary pipette. The craniotomy was covered with Kwik-Sil (Kwik-Sil Silicone Elastomer, World Precision Instruments Inc., USA) and the skin sutured (Supramid 4/0, Resorba Medical GmbH, Germany).

2.3.3 Medial Forebrain Bundle Electrode Implantation

The implantation of a bipolar stimulating electrode in the MFB was performed on rats between 12 and 18 weeks of age (N=10), and between 400- and 500-gram bodyweight. For animals in the control group (N=6), this implantation was the only surgical procedure the animal underwent.

First, a craniotomy for the electrode was made with a 1 mm burr with the following coordinates from bregma: AP: -2.8 ML: +1.7, and dura was exposed and punctured. Five additional skull holes were drilled for anchor screws, two left of the midline, one to the right of the midline and two posterior to lambda. A bipolar stimulating electrode (MS303/3-B/SPC, Plastics One, Canada) was lowered +8 DV into the brain, measured from dura. After implantation, the electrode and craniotomy were carefully covered using Kwik-Sil. Super-Bond (Super-Bond C&B, Sun Medical Co., Ltd., Japan) was then applied to the skull, anchor screws and the Kwik-Sil, to strengthen the bonding between skull and cement. Self-curing dental cement (Meliodent, Kulzer GmbH, Germany) was mixed and applied around the electrode and on the skull and anchor screws. When the cement had dried, sharp edges were removed with a drill. If the dental cement itself was not sufficient to cover and close the entirety of the wound, sutures were applied where necessary.

2.3.4 Fiber-Optic Cannula Implantation

For the experimental animals, a bipolar stimulating electrode targeting the MFB and dual fiber-optic cannulas (DFC_200/245-0.37, Doric Lenses, Canada) with a distance of 1 mm between the two fibers were implanted when the animal was between 12 and 15 weeks of

age and between 400- and 500-grams bodyweight. The fibers were etched in 48% hydrofluoric acid at SINTEF (Trondheim) by Ida V. Rautio to increase the light spread and reduce brain tissue heating (Kosoglu et al., 2011), and manually inspected before implantation. Every cannula implant was tested and calibrated to the desired laser intensity (~30 mW). The specifications which resulted in the correct amount of light emitted was noted for every implant, and a list of which implants that were coupled with which animal was saved for later reference (section 2.5.2).

When implanting the dual fiber-optic cannulas, the craniotomy previously performed for the viral injections was reopened, removing any residues of Kwik-Sil and drilling off potential skull formations that had developed since the previous surgery. The fiber-optic cannulas were lowered at a 20 degree angle and the coordinates used were the following from bregma: AP: +3.3-3.2, ML: 0 (since the diameter of the fiber-optic cannulas were 1 mm, their final target would then be ML: ±0.5) and DV: +2.0-2.1 measured from dura. After implantation of the cannulas, a bipolar stimulating electrode targeting the MFB was implanted, using the same procedure and coordinates described in the previous paragraph. Kwik-Sil was applied to cover both craniotomies for the fiber-optic cannulas and the bipolar electrode. Super-Bond was applied on top of the Kwik-Sil, the screws and on the base of the fiber-optic cannula implant, and both implants were cemented in place with dental cement.

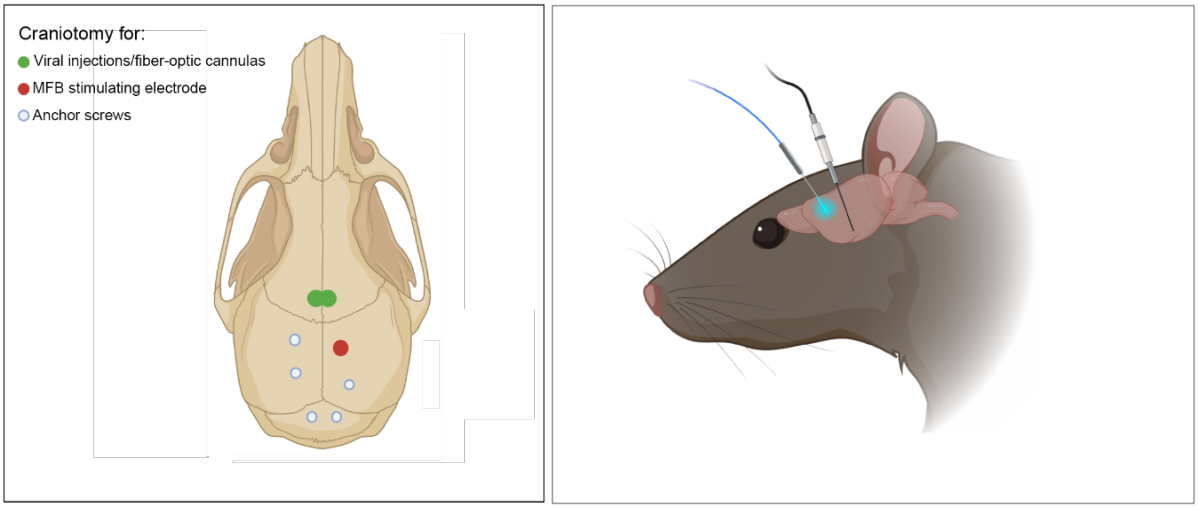

Figure 2.1 a) Craniotomy and implantations. Overview of the craniotomies, with craniotomy for viral injections and the fiber-optic cannulas in green, bipolar stimulating electrode targeting the MFB in red and anchor screws in white. The craniotomy for viral injections and the fiber-optic cannulas were only performed on the animals in the experimental group. **b)** Illustration of the implants, with the fiber-optic cannulas anterior to the bipolar stimulating electrode. The figures are only meant for illustrative purposes and do not use exact coordinates. The figures are made with BioRender.com

2.3.5 Post-Surgical Care

After surgery, the animal was kept in a heating chamber at 30 ºC until it recovered from anesthesia and was moved back to its cage. Temgesic was injected for pain relief 6-8 hours after the initial injection. 24 hours after the initial injection, the animal received Metacam, and an additional dosage of Temgesic if it showed any signs of being in pain. If the animal showed signs of pain the following days, it received additional Metacam and Temgesic according to the established guidelines. The animal was given a minimum of five days to recover from surgery before the effect and placement of the stimulating electrode was tested. The aforementioned habituation sessions to the performer chamber were completed during the initial post-operative recovery days before the testing of the stimulating electrode occurred. If the animal needed more time to recover and was unable to be habituated to the performer chamber within the first five days, testing of the electrode would be delayed until the habituation sessions were completed such that no rewarding sensations would be associated with the performance chamber before the actual experiment was to be initiated.

2.4 Apparatus

2.4.1 Experiment Box and Equipment

The experiments were performed in a dimly lit room. It took place in a plexiglass box (100x40 cm). The box contained two compartments, referred to as the observation chamber (40x40 cm) and the performer chamber (60x40 cm) (fig. 2.2). For this specific experiment, the observer chamber was used for habituation and electrode testing in order to keep the treatment of the animals identical across the different experiments in the paradigm. For the actual experiment sessions in this study, only the performer chamber was used.

Two ping-pong balls were mounted on top of a metal rod inside the performer chamber, with an LED inside the balls to allow for them to light up at task-specific times. It was designed so that when the ball was pushed, the metal rod would touch copper tape underneath the box which the Rapsberry Pi controlling the task would register and record a time stamp for when the ball was pushed. The Raspberry Pi was connected to a Raspberry Pi camera (Pi NoIR Camera V2, Raspberry Pi Foundation, UK), and to a pulse stimulator (Master 9, Microprobes, USA). The pulse stimulator was in turn connected to a stimulus isolation unit (ISO-Flex, Microprobes, USA), delivering the stimulating pulses to the animal

via the electrodes implanted in the MFB. Infrared lights were used for illumination for the video recording.

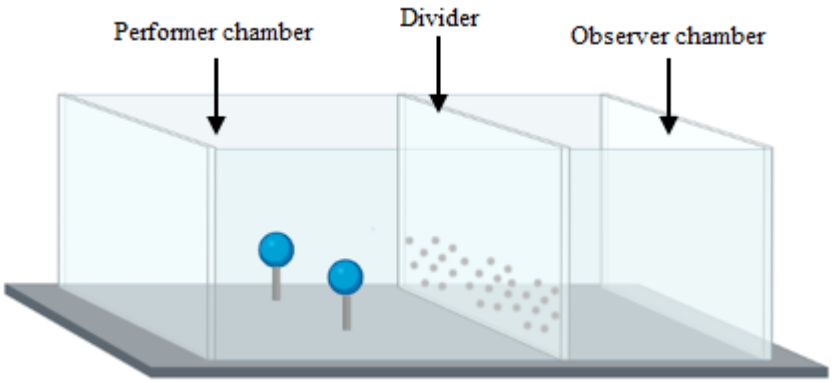

Figure 2.2 The experimental box in which the experiment was performed. For the current experiment, the observer chamber was used for habituation and electrode testing, while the performer chamber was used for the actual experimental sessions. The figure is made by Devika Kurup with BioRender.com.

2.4.2 Laser Set-Up

For the optogenetic condition, we used two 473 nm DPSS lasers (150 mV, Shanghai Lasers & Optics Century, China). The set-up consisted of two laser control apparatuses, which controlled two laser emitters. The light emitted went through adjustable dimmer wheels and reflected off two mirrors, one for each laser, which in turn sent the light into two separate collimators, devices that narrows the beams of light. A 4-meter-long dual fiber-optic silica patch cord (Doric Lenses, Canada) was connected to the collimators through SMA-connectors at one end and could be connected to the fiber-optic cannula implants in the other end. The fiber-optic patch cord stretched from the collimators to the performer chamber and was suspended using elastic bands on a custom rig. Black walls and lid made of black hardboard (TB4, Thorlabs, Inc., Germany) were built for this specific set-up to cover the lasers.

2.4.3 Experiment Script

The experiment sessions were performed using a custom-written script on the Raspberry Pi written in Python 2 by Benjamin Adric Dunn. The script controlled the timing of the rewarding stimulation, lighting of the LEDs inside the ping-pong balls, ran the recording camera, saved the resulting files after each session, and had the option of running the task automatically or giving the experimenter manual control over all these aspects. In

automatic mode, the first ball would light up at a random time interval between 3 and 30 seconds after the initiation of each new trial. If the first ball was pushed while the light was on, the light would turn off and the light in the second ball would turn on. If this second ball was pushed while the light was on, the light turned off and the rat received a rewarding stimulation. If the balls were not pushed within 30 seconds after lighting up, the light would turn off and no rewarding stimulations would be given. If the first ball was not pushed, it was considered a missed trial, and if the first but not the second ball was pushed, it was considered a failed trial. When the lights turned off, either due to completing a successful trial or an unsuccessful trial, the script would immediately start a new trial with a new random time interval before the light in the first ball lit up again. Starting the script would initiate a video recording, and every session would result in a video and a .txt-file with the raw output for each given session.

2.5 Behavioral Protocols

2.5.1 Electrode Testing Protocol

For the rat to be used in the experiments, it was necessary that the MFB implant hit the intended target in the brain during surgery, which would result in the rat experiencing rewarding sensations (Hernandez et al., 2006) when stimulated. To test the function of the electrode, we tested if we could make the rat interested in an arbitrary object, specifically a pen, as a result of manually giving stimulations at different levels of strength.

The electrode cord was coupled to the rat's electrode implant while the animal was placed on the researcher's lap. To avoid stress, the animal was never restrained, and the cord was usually connected while the rat was eating a treat and thus reducing its head movements to allow for cord attachment. The rat was gently placed inside the observation chamber together with the pen. When the animal groomed, usually considered as a sign of relaxation, the script was started which initiated a video recording. Whenever the animal approached the pen, it received manual stimulations by the experimenter. The starting intensity for the stimulations were set to 20 mV. The intensity was then adjusted upwards with an interval of 2 mV per adjustment. If the animal's response indicated reward, we tested if we could find a range with stable responses and without side-effects, which was then used for the experimental sessions. The intent was to trigger motivation but not overexcitation by single stimulations, as stimulations with too high intensity could lead to the animal being in a

chronic overexcited state after repeated stimulations. If the reaction was too weak the animals would usually show initial interest and some investigation of the pen through orientation and sniffing, but not engage any further with the object. If the reaction was too high, they commonly displayed jolting behavior in response to stimulation. An example of a suitable reaction would be that the rat was interested enough to lift and actively interact with the pen after a single stimulation. The intensity that typically produced this response or similar behaviors was found to be 24-28 mV. When a suitable stimulation intensity was found, we additionally tested the effect of MFB stimulations on the animals in the experiment group with light stimulation through the fiber-optic cannulas. This was done to ensure that the optogenetic inhibition of neurons in the ACC would not influence the experience and physical expression of rewarding sensations.

In some cases, the animal would, in addition to experiencing reward, display a motor artifact. These would show as a consistent movement or twitch every time stimulation was given, typically towards their left side as the rats were implanted in the right hemisphere. In other cases, the stimulation resulted in aversion to the object. The animals that showed side-effects hindering learning or task performance were excluded from the experiment. Ideally, we sought to avoid the use of animals with motor artifacts, but as a result of restricted time and available fiber-optic cannula implants, one such animal (# 27037) was included as it still showed clear signs of reward, and the motor artifacts did not impair the animal's ability to move and engage with the object.

2.5.2 Protocol for Optogenetic Inhibition

The goal of the optogenetic protocol was to effectively and reversibly inhibit neurons in the ACC during the total of 30 minutes in each training session, without damaging the brain tissue. The laser control apparatuses were turned on 10 minutes prior to use to allow for sufficient warm up time. Before every session, the laser intensity for each of the fibers were adjusted with the dimmer wheels to correspond to the tested intensities pre-surgery for each of the fiber-optic cannula implants, using an optical power meter (PM 100D, Thorlabs, Inc., Germany) in dim light. The intended light emitted to the brain tissue was ~30 mW (±1-2 mW) in each hemisphere. To create pulsating light, an Arduino was connected to the laser control apparatuses with a program to make the lasers pulsate at a 60 Hz frequency. All work done with the lasers was performed with safety glasses to avoid laser damage.

To ensure that this laser intensity and pulsating frequency lead to a functional and reversible inhibition of the neurons, the protocol of using ~30 mW and 60 Hz pulsation was developed and tested by Ida V. Rautio (Appendix B). Three animals were used for this purpose. These animals were injected with the viral vector, and later implanted with the fiber-optic cannulas and a Neuropixels 1.0 probe (Imec, Belgium) to allow for simultaneous neural recordings of the activity during the optogenetic silencing, to verify the efficacy of the virus and the specific protocol.

2.5.3 Experimental Protocol

The goal of the experiment was to investigate whether optogenetic inhibition of neurons in the ACC in a rat would impact the learning of a reward-driven, non-social task. Four of these experiments were performed by my co-supervisor Ida V. Rautio, two by my colleague Devika Kurup and the remaining four by the author.

To make testing conditions consistent across experimental groups, we turned on the laser control apparatuses before all sessions as they produced a humming sound. After attaching the electrode cord and, for experimental animals, the fiber-optic patch cord as well, the animal was carefully placed inside the performer chamber. We waited until the animal groomed before turning on the lasers and starting the script. A stopwatch was used to track the duration of the session. When training the animals, we used the principles of shaping, rewarding intermittent steps and gradually decreasing the number of rewards. The goal was to make the rat perform the sequence of tapping the two ping-pong balls in the right order when lit, with only a single rewarding stimulation following the push to the second ball (fig. 2.3). The experiment protocol followed specific steps:

1. The animal would receive stimulations when approaching the balls irrespective of which ball it approached. At this step, no lights were used.
2. The balls were lit, one at a time. The animal would only receive stimulation if it approached the ball that was lit.
3. The animal would only receive stimulation if it pushed the ball that was lit. When the first ball was pushed, the light was turned off and the second ball lit up, imitating the whole correct sequence but with rewards for pushes to the first ball.
4. When the animal consistently toggled between the balls for approximately 10 minutes, pushing them in the right order, we would stop stimulating at the first ball.

5. If the animal continued to toggle and push the balls when not stimulated at the first ball, we would switch to automatic mode. Then the animal had a time limit of 30 seconds, after which the light would switch off if not pushed, and a new trial started.

If the animal failed to perform on a certain step on multiple consecutive trials, we would go back to the previous step. For instance, if the rat stopped pushing the first ball when it did not result in reward (step 4) and this consisted over a time span of approximately two minutes, we would go back to rewarding pushes at the first ball (step 3) again.

This shaping and training were performed in sessions lasting 30 minutes over eight days, with a one-day break after the fourth day, resulting in seven sessions in total. This number of sessions was chosen as all three experimenters had found that most rats reached asymptotic performance within seven sessions when training performer rats for the observational learning experiments. Leaving animals implanted for prolonged periods carried a risk of complications, so the decision of a cut-off at seven sessions were balanced between lowering the risk of complications and giving the animals the possibility to display learning.

The sessions took place at approximately the same time of day on consecutive days for each animal, but the time of day could vary across animals. If the animal got >75% correct trials on a fully automatic session before the seven sessions were completed, it was considered to have reached the learning criterion. The number of sessions needed to reach learning criterion was noted, and the experiment was ended after a test session on the following day.

When designing this experiment, we were unaware of any potential effects of temporarily inhibiting neurons in the ACC. We speculated whether there could be any side effects of the inhibition of neurons in the ACC that could reduce the performance of the animals and leave the animals unable to display learning. For example, secondary motor cortex (M2) is bordering to Cg1, and if there were any unexpected virus spread and off-target effects by the optogenetic manipulations, the animal's motor function could be impaired which in turn could disrupt the performance without directly disrupting the learning ability per se. For this reason, we chose to have an extra test session the day after they reached the learning criterion or after the seventh and last session, in which neurons in the ACC of the rats was not optogenetically inhibited. This session was conducted without any additional stimulations given by the experimenter; thus, it was a completely automated, script-driven session. We included this test session for both groups to have comparable conditions.

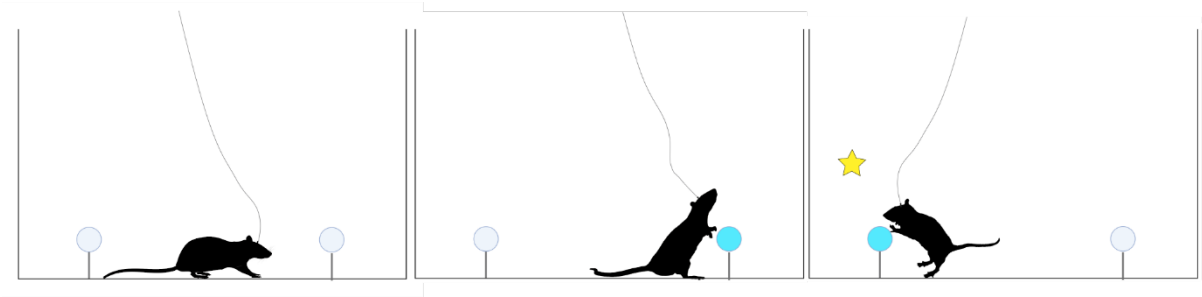

Figure 2.3. **Illustration of the task.** Initially, no lights are on, and this is considered a time-out period during which no actions by the rat will result in reward. Then, the first ball lights up and the rat needs to push the ball within 30 seconds. A push results in the light turning off in the first ball and on in the second ball. A push of the second ball within 30 new seconds results in a rewarding stimulation, illustrated here by a star, and the light turns off and a new trial begins. If the first ball is not pushed within 30 seconds, the light turns off and it is considered a missed trial. If the second ball is not pushed within 30 seconds, it is considered a failed trial.

2.6 Transcardial Perfusion

During the development of a working optogenetic silencing protocol, it was discovered that the prolonged continuous laser exposure could lead to necrosis of the brain tissue. While necrosis was not seen in any of the animals used in the experiment, we followed a strict protocol of perfusion 24-26 hours after the last laser exposure for the first four experimental animals. This was done to minimize the impact time could have on varying degrees of glial scarring. After it was determined that the final optogenetic silencing protocol used in this experiment did not result in any damage to the brain tissue, this strict protocol of perfusion 24-26 hours after last exposure was replaced with a protocol of performing the perfusions within a few days after the experiment ended. This was done in order to minimize the impact time could have as a potential variable for potential differences in virus expression after completing the immunohistochemistry protocol, but still allow for some leeway in terms of scheduling the perfusions after each experiment. The control animals which had not received any viral vector injections did not follow this protocol and were typically re-used as performer rats in a separate observational learning experiment before being euthanized. This was done to maximize the use of all animals and minimize the number of animals needed for multiple ongoing experiments.

After an experiment was completed, the rat was euthanized by way of transcardial perfusion. Before perfusion, a Ringer's solution (3.35 mM KCl, (Merck KGaA, Germany), 145 mM NaCl (VWR International Ltd., USA), 2.28 mM NaCHO3 (Merck KGaA, Germany)) and 4% depolymerized paraformaldehyde (PFA, pH 7.4 (Merck KGaA,

Germany) in 0.125 M phosphate buffer (PB, Merck KGaA, Germany) was prepared. The animal was weighed and then anesthetized in an induction chamber with 5% isoflurane. When unresponsive, the rat received an intraperitoneal injection with a weight-appropriate dosage of pentobarbital (Exagon vet., 400 mg/ml, Richter Pharma Ag, Austria) before being placed back in the induction chamber with 2% flowrate of isoflurane. When the respiration noticeably slowed down, the rat was transferred to a down-ventilated surgery table and laid on its back on a grid in a tray. Reflexes were tested pinching the skin between the toes of the paws with forceps. When no reflexes were exhibited and the rat was at a terminal stage, a small incision was made laterally beneath the ribcage, exposing the diaphragm. From this incision, two diagonal incisions were made in the ribcage and the sternum lifted with a hemostatic clamp, exposing the heart. With a perfusion pump (Peri-Star Pro, World Precision Instruments Inc., United Kingdom), Ringer solution was infused into the left heart ventricle through a 21 g syringe needle (Sterican, B.Braun Melsungen AG, Germany). A small incision was immediately made in the right artery using surgical scissors, allowing the Ringer solution to rinse the circulatory system for blood. When the Ringer solution exiting the artery was sufficiently cleared of blood, and with a minimum of 150 mL used, the tube to the Ringer solution was disconnected from the pump. Keeping the syringe in the left ventricle, the tube to the PFA solution was connected to the pump. PFA solution was infused into the circulatory system of the animal until fixating tremors diminished, with a minimum of 150 mL. The animal was decapitated, and the skin and muscles around the skull removed. The brain with surrounding skull was kept in PFA solution overnight to sufficiently fixate the brain. The following day, the skull was carefully removed using rongeurs. The fixated brain was kept in dimethyl sulfoxide (DMSO, VWR International Ltd., USA) in a fridge (+4 ºC) until further tissue processing.

2.7 Histology

2.7.1 Sectioning

The fixated brains were sectioned using a microtome (Microm HM430, Thermo Fisher Scientific, USA). The microtome stage was straightened using leveler and cooled down to -10 ºC, and a layer of sucrose (VWR International Ltd., USA) dissolved in 0.125 M PB was applied to protect the stage. The stage was then cooled down to -40 ºC. Before mounting the brain onto the platform, a straight cut was made through the cerebellum, making an even base on which the brain could stand. The brain was sectioned in a coronal

plane. The brain was set on the sucrose base and its angle visually inspected, confirming that the midline was perpendicular to the microtome stage. Additional sucrose was added to the base to keep the brain stable, and the brain covered in dry ice to lower the temperature.

When the brain was thoroughly frozen, it was sliced into 40 µm sections. The brains were sectioned in three series, the first series being mounted immediately in Tris(hydroxymethyl)aminomethane (Tris, Merck KGaA, Germany) on Superfrost slides (Thermo Fisher Scientific, USA). The two remaining series were collected and stored in section tubes in DMSO in a freezer (-25 ºC). After every second series, the brain was covered in dry ice again to keep the brain from defrosting. For the animals with fiber-optic cannulas and viral injections, the collection of sections began after the olfactory bulbs and continued until approximately 600 µm after the electrode trace was collected. For control animals, collection began when the hippocampus was visible, and continued until approximately 600 µm after the electrode trace.

2.7.2 Nissl Staining

To visualize anatomical borders necessary for confirming the placement of the cannulas and electrode implants, the first series from all sectioned brains was stained following a Nissl protocol. Superfrost slides with mounted brain tissue sections were placed in a slide holder. The sections were dehydrated through being dipped 10 times into 50%, 70%, 80%, 90%, 100%, 100% and 100% alcohol (KiiltoClean AS, Norway), before they were placed in Xylene (Mixture of Isomers, VWR International Ltd., USA) for 2 minutes for clearing and demyelination to reduce background staining. After rehydrating the sections following the same protocol in reversed order, the sections were quickly rinsed in water and then placed in a Cresyl Violet solution (0.5 g Cresyl Violet Acetate, Merck KGaA, Germany) in darkness on a shaker for 3 minutes. The sections were rinsed in water and a mix of 70% alcohol and acetic acid (98%, Merck KGaA, Germany) until excess color was washed off. The slides were then turned upside down in the slide holder and placed in the Cresyl Violet solution for 2 additional minutes, before the same procedure with rinsing off excess color was performed, until the desired level of contrast was reached. The sections were dehydrated again and placed in Xylene for minimum 5 minutes, and the slides then cover slipped using Entellan (Merck KGaA, Germany).

2.7.3 Immunohistochemistry

The virus expression was visualized using immunohistolochemical labelling of mCherry. A solution containing 0.01 M phosphate buffer saline (PBS, Merck KGaA, Germany) + 3% Triton (Merck KGaA, Germany) and a solution containing PBS + 0.3% Triton + 3% bovine serum albumin (BSA, Merck KGaA, Germany) were prepared in advance by the group's technician Merethe Andresen and stored in a freezer (-20 ºC). Triton was added to make the cells more permeable, and the BSA was added as a general blocking agent, blocking the potential secondary antibody sites. When possible, the sections were covered with a lid to avoid unnecessary bleaching as a result of light exposure.

The second series with sectioned brain slices were washed in 0.01 M phosphate buffered saline for 3 x 5 minutes while being placed on a shaker with 60 rpm. They were then washed 2 x 10 minutes in a solution containing 0.1 M PBS + 0.3% Triton, to remove lipids and ease antibody binding. The tissue sections were kept in a 4995 µL PBS + Triton and 5 µL red fluorescent protein (RFP) antibody (5F8, ChromoTek Cat# 5f8-100, RRID:AB_2336064, dilution 1:1000, Chromotek GmbH, Germany) solution on a shaker (60 rpm) in the dark in a fridge for 24 hours. Following this labeling phase with the primary antibody, the sections were washed for 2 x 5 minutes in the solution containing PBS and Triton. They were then incubated with secondary antibodies in a solution containing 4995 µL of the prepared solution containing the blocking agent and 5 µL secondary antibody (Goat Anti-rat IgG (H+L) (Alexa Fluor 546), Thermo Fisher Scientific Cat# A-11081, RRID:AB_2534125, dilution 1:1000, Thermo Fisher Scientific, USA) for 1 hour on a shaker (60 rpm). Finally, the sections were washed for 2 x 10 minutes in PBS on a shaker. If necessary, they were kept overnight in PBS before they were mounted in PBS on gelatin-coated (Chemi-Teknik AS, Norway) Polysine slides (Thermo Fisher Scientific, USA) prepared by our lab's technician. The slides were left to dry on a heating plate in the dark, covered with a lid covered aluminum foil, and cover slipped when dry using Toluene (VWR International, Ltd., USA).

2.7.4 Microscopy and Scanning

When the cover slipped slides were dry, excess Toluene or Entellan was removed by scraping with a razor blade and wiping with 70% ethanol. The slides were scanned using Axio Scan.Z1 (Carl Zeiss AG, Germany) to determine the implant sites and spread of the injected viral vector. Tissue stained with Cresyl Violet were scanned with brightfield at 20x

magnification. Tissue stained with fluorescent antibodies were scanned at 20x magnification with fluorescent detection at 546nm wavelength, suitable to display the viral vector mCherry tag.

2.7 Data Analysis

2.7.1 Processing of Raw Data

The custom-built script on the Raspberry Pi that controlled the experiment (section 2.4.3) created a video file and a .txt file for every experimental session. This raw data-file contained the time when the session began, when the lights turned on and off and when a rewarding stimulation was given. From the fully automated sessions, the raw data was analyzed using a custom-written code, written in Python 3 by Ida V. Rautio and later refined by Michael Staff Larsen. The output after running the raw data-file with the analysis script included the number of correct/missed/failed trials, proportion of trials that were correct, the latencies and average trial lengths, and the length of the session. This output was used for statistical analyses.

2.7.2 Perseveration Score

The raw-data file from the experimental sessions only included the ball pushes during an ongoing trial, not including the activity during the time-out periods. To get an indication of the animals' perseverative behavior, the experiment videos from the learning sessions were analyzed manually by the author, counting every time the animal pushed the second ball outside a trial. The animals were only given a score if the ball was visibly moved by the push, and continuously pressing the ball for prolonged periods did not result in an additional score. As the animals sometimes performed the whole sequence correctly during the time-out period, only pushes to the second ball that did not follow an unregistered push to the first ball was counted. Hence, pushes to the second ball either after receiving a reward or pushes without pushing the first ball first was seen as the animal's perseveration score.

2.7.3 Statistical Analyses & Data Visualization

Statistical analyses were carried out in SPSS Version 27.0.1.0 (Statistical Package for Social Sciences, IBM Analytics). As the sample size was small, with a total of 10 data points (experimental group n = 4, control group n = 6), the sample lacked statistical power. With a

sample this size, normality tests have little power to detect non-normality and is considered futile (Ghasemi & Zahediasl, 2012). Thus, non-normality was assumed, and the experimental groups were compared using a two-tailed non-parametric test, the Mann-Whitney U test, unless otherwise specified. The statistical significance level, alpha, was set to α =.05. The mean value (M) and standard deviation (SD) was reported for every measure.

The groups were compared with a chi-square test on the binary score of whether or not they reached the learning criterion of >75% correct trials in a fully automated session within the seven sessions. The few animals that did not reach this learning criterion within seven sessions were excluded from subsequent statistical analyses, as to get a representative comparison of accuracy and time spent on the task. This excluded two control animals, resulting in n = 4 in the experimental group and n = 4 in control group. The descriptive statistics from the non-learners' test session is noted in appendix B. For the animals that did reach the learning criterion, henceforth termed learners, the number of sessions needed until they reached the criterion in each group was compared.

From the learners, the groups were compared on the scores from the session in which they reached the criterion, henceforth termed the learning session. Since all the animals underwent an additional test session, without optogenetic silencing of the ACC, the data from the test session was also compared. The groups were compared on the following measures from both sessions: 1) proportion correct trials, henceforth termed accuracy, 2) the latency from the trial began until the first ball was hit, 3) the latency from the first ball was hit until the second ball was hit and a reward was given, 4) average trial length, 5) the perseveration score. The perseveration score was only compared for the learning session. Additionally, the difference in accuracy and latency from the first to the second ball push between the learning session and the test session for each group was compared with Wilcoxon Signed-Rank test, a non-parametric test for comparison of two dependent samples.

The data was visualized with box plots with overlaying swarm plots, constructed in Python 3.8 using the libraries Seaborn (Waskom, 2021) and Matplotlib (Hunter, 2007). The box plots indicate the median and the upper and lower quartile of the data, with whiskers to indicate outliers. The swarm plots show the individual data points. The plots were stylized in Adobe Illustrator® (Adobe Systems, U.S.A).

2.7.4 Visualization of Virus Expression and Confirmation of Implant Sites

All the scans were compared against the Rat Brain by Paxinos & Watson (2007). The placement of the electrode was visually inspected, but as the confirmation of electrode function came from the animals' behavior, the histology was mainly used as verification that the implant had reached the intended target. In the animals that did not have a working electrode, the histology was used to guide subsequent implantations. The placement of the virus expression and the fiber-optic cannulas were estimated using the Rat Brain by Paxinos & Watson (2007). To get a visual 3D-presentation of the virus expression, a software named TRACER, developed by the Whitlock group, was used (Paglia et al., in progress). This program was originally developed for estimating the location of implanted probes in the rat brain but was modified for the additional visualization of viral expression. The histology figures were made with Adobe Photoshop® (Adobe Systems, U.S.A).

4. RESULTS

3.1 Histology

The brain slides were processed with a Nissl protocol and the virus expression in the experimental group was visualized through immunohistochemical labeling of mCherry. Through visual inspection and comparison with the brain atlas the Rat Brain by Paxinos & Watson (2007), the location of the expressed virus was confirmed to be in Cg 1 & 2, with some spread towards the prelimbic cortex (PL). This is in accordance with the wider definition of ACC delineation, as described in paragraph 1.2.2. In addition, there were some spread to secondary motor cortex (M2). For all the animals in the experimental condition, a 3D visual representation was made with the TRACER software (Paglia et al, in progress) (Fig 3.1).

The placement of the fiber-optic cannulas was confirmed to be hitting the target area, in the center of the viral injection and in the Cg 1 & 2 region (Fig. 3.2). In one animal (#27399), some necrotic brain tissue in the Cg1 and M2 was discovered, but was confined mainly to M2 and did not encroach visibly into Cg1. As the animal displayed no behavioral changes following surgery, the animal was kept for analyses. The placements of the stimulating electrodes targeting the MFB were mainly verified through the behavioral verification of the electrode test (paragraph 2.5.1) but were confirmed through visual inspection and comparison with the Rat Brain by Paxinos & Watson (2007) (Fig. 3.3).

3.2 Behavioral Data Analysis

3.2.1 ACC Inhibition does not Eliminate Learning

The learning criterion was set to >75 % correct trials on a fully automated 30 minutes session within seven sessions. All the animals in the experimental group (100%, n = 4) reached this criterion, in contrast to only two thirds of control group (67% of n = 6). A Chi-Square using a binary score of 0 and 1 for not learning and learning, respectively, revealed no significant difference, but there was a trend towards more learners in the experimental group ($\chi 2$ (1, N = 10) = 3.6, p = .058).

Contrary to our first hypothesis, the number of sessions needed to reach the criterion in the experimental group (n = 4) (M = 4.25, SD = 0.957) did not differ from the control group (n = 4) (M = 3.50, SD = 0.577), (U = 4.0, p = .343) (fig. 3.5a). This indicates that

temporary inhibition of neurons in the ACC during training sessions did not impact learning speed on a sensory-motor task such as the one used in this project.

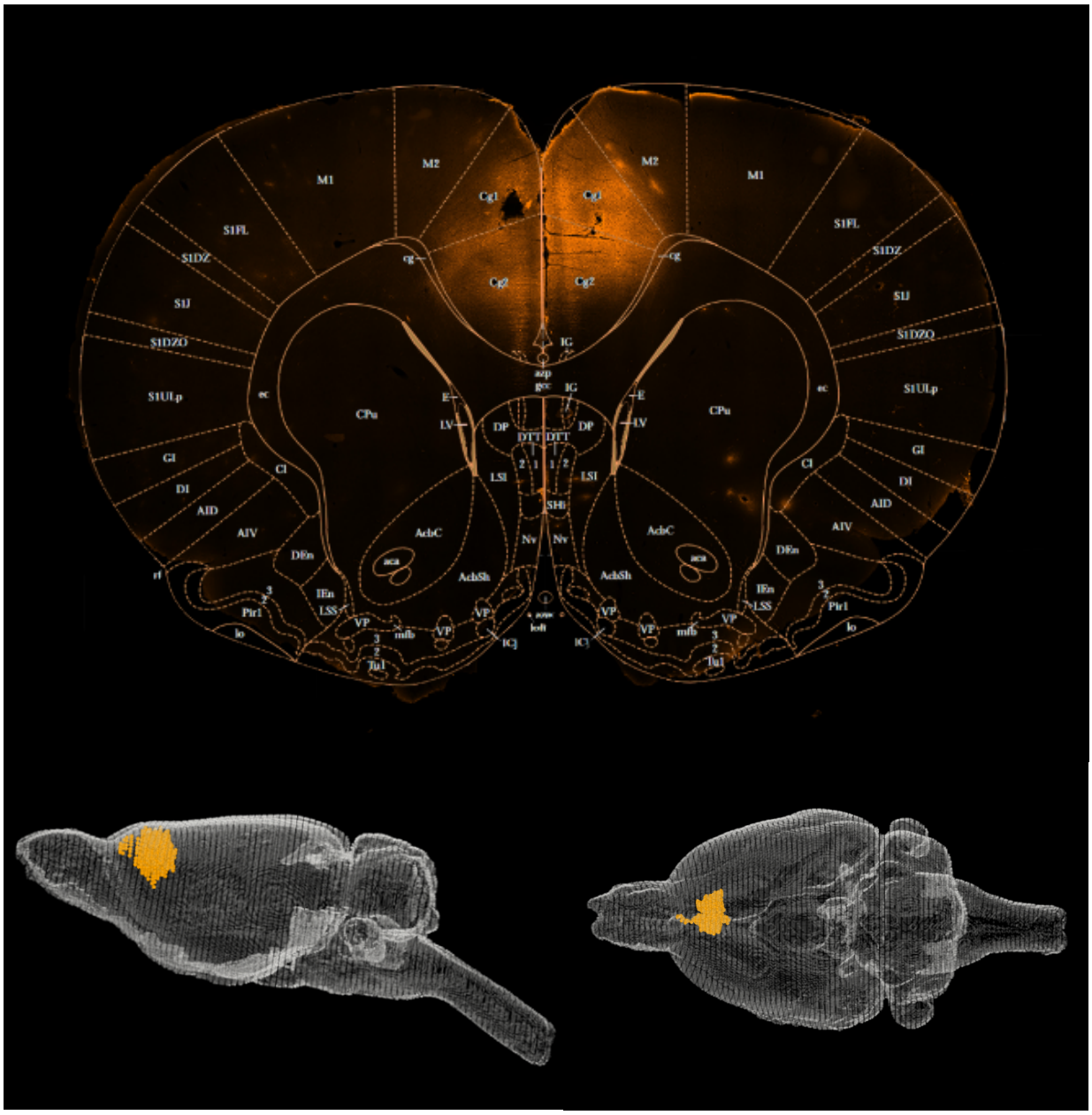

Figure 3.1). Localization of virus expression. Example of the fluorescent immunohistochemical labeling of mCherry in rat #26991, Lemminkäinen, with an overlay from the Rat Brain by Paxinos & Watson (2007), at anterior-posterior coordinates +2.28 from bregma. The bottom images are examples of the visual representation of the virus spread in rat #26991, made with TRACER, seen from the side and from above, respectively.

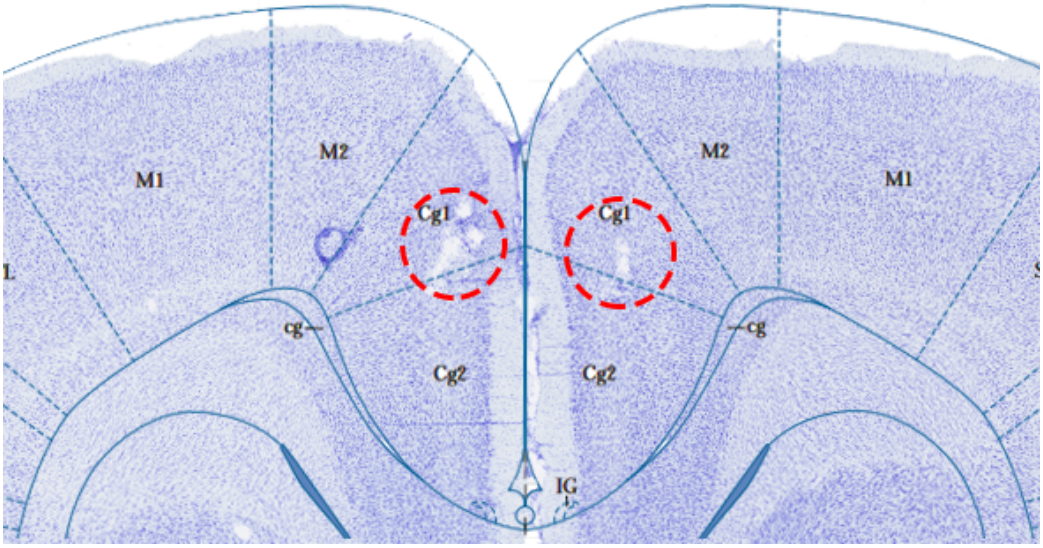

Figure 3.2. Localization of the fiber-optic cannulas. Example of Nissl-stained brain tissue from rat #26991, Lemminkäinen, showing the traces of the implanted fiber-optic cannulas targeting cingulate cortex 1&2 (Cg 1&2), with an overlay from the Rat Brain by Paxinos & Watson (2007), at anterior-posterior coordinates +2.28 from bregma.

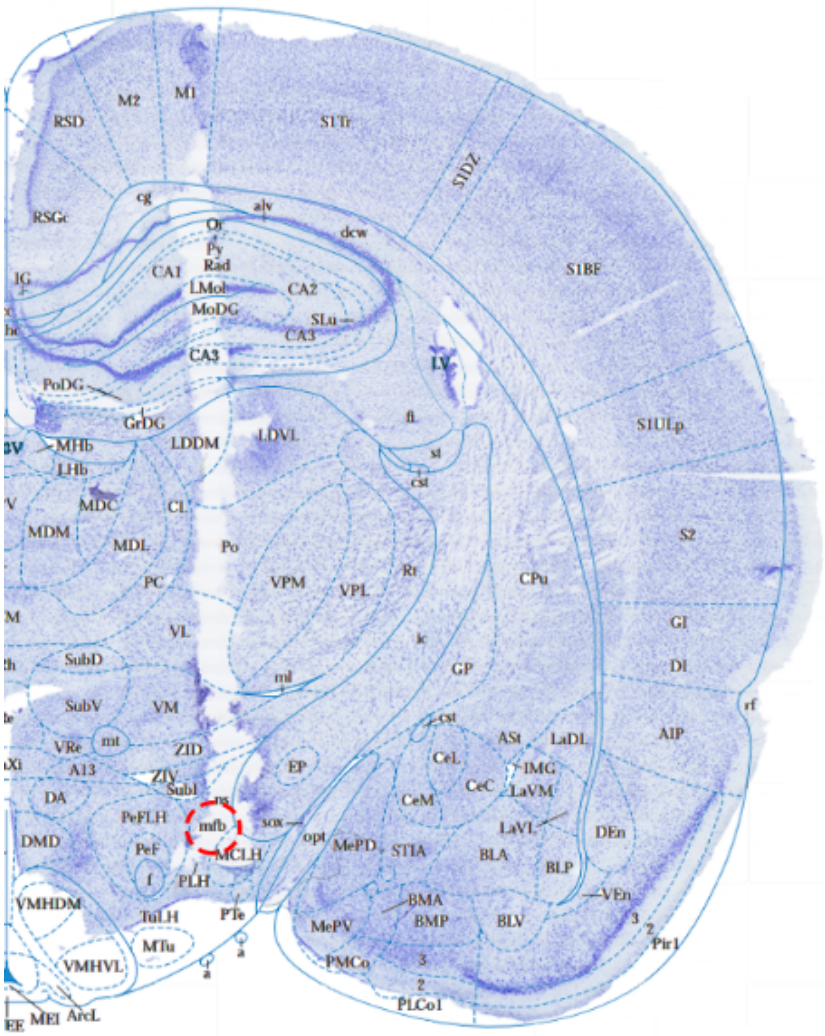

Figure 3.3 Localization of the bipolar stimulating electrode. Example of Nissl-stained brain tissue from rat #26991, Lemminkäinen, showing the localization of the bipolar stimulating electrode targeting the medial forebrain bundle (MFB), with an overlay from the Rat Brain by Paxinos & Watson (2007), at anterior-posterior coordinates -2.76 from bregma.

3.2.2 ACC Inhibition does not Reduce Accuracy

The data from the learning session (experimental group n = 4, control group n = 4) revealed no significant difference on accuracy (U = 5.000, p = .343) between the experimental group (M = 85.61, SD = 6.90) and the control group (M = 90.69, SD = 7.766) (fig. 3.4a). This is contrary to the second hypothesis that temporary inhibition of neurons in the ACC would reduce the accuracy during task performance.

Whereas the groups did not differ significantly in the learning session, the experimental group was found to have significantly lower accuracy (M = 57.40, SD = 23.30) compared to the control group (M = 92.68, SD = 8.86) in the test session (U = 0.0, p = .029). (fig. 3.4a) The decrease in accuracy in the experimental group from the learning session to the test session when the ACC was not inhibited is further described in section 3.2.4.

3.2.3 ACC Inhibition Increases Performance Speed

Performance speed was divided into three components, the average latency from when the first ball lit up until it was pushed, the average latency from pushing the first to the second ball, and the average sum of these two (total trial length). During training it became apparent to the experimenters that many animals were able to exceed 75% correct trials without understanding the light cues, but instead repetitively perform the ball-pushing sequence irrespective of the light status. The latency from when the first ball lit up until the ball was pushed could therefore vary substantially. Thus, the latency from the first to the second ball push was considered the most informative measure of performance speed, but analyses were performed for all three measures.

In the learning session (Table 3.1), the two groups did not differ significantly on either latency from when the first ball was lit up to when it was pushed (U = 5.0, p = .486) (fig. 3.4b) nor on the average trial latency (U = 4.0, p = .343) (fig. 3.4d). However, the animals in the experimental group spent significantly longer time from the first to the second ball push (U = 0.0, p = .029) (fig. 3.4c).

Performance Speed Learning Session	Latency 1st ball (seconds)	Latency 2nd ball (seconds)	Average Trial Length (seconds)
Experimental Group (N=4)	*M* = 12.03 *SD* = 3.07	*M* = 4.10 *SD* = 1.22	*M* = 16.13 *SD* = 4.14
Control Group Learners (N=4)	*M* = 9.11 *SD* = 3.88	*M* = 2.69 *SD* = 0.46	*M* = 11.80 *SD* = 4.33

Table 3.1 Overview of performance speed from learning session. The control group was consistently quicker to perform the task than the experimental group in the learning session. This difference was only significant for the latency from the first to the second ball push.

Similarly, in the test session (Table 3.2), there were no significant differences between groups for the latency from when the first ball lit up to when it was pushed (U = 1.0, p = .057) (fig. 3.4b), nor for the average trial length (U = 1.0, p = .057) (fig. 3.4d), although there was a trend towards a higher latency for both measures in the experimental group. As for the learning session, the latency from the first to the second ball push differed significantly between the groups, with the experimental group taking longer between first and second ball hit (U = 0.0, p = .029) (fig. 3.4c).

Performance Speed Test Session	Latency 1st ball (seconds)	Latency 2nd ball (seconds)	Average Trial Length (seconds)
Experimental Group (N=4)	*M* = 18.31 *SD* = 6.67	*M* = 4.15 *SD* = 0.52	*M* = 22.46 *SD* = 7.16
Control Group Learners (N=4)	*M* = 7.48 *SD* = 3.62	*M* = 2.49 *SD* = 0.80	*M* = 9.96 *SD* = 4.19

Table 3.2 Overview of performance speed from test session. The control group was consistently quicker to perform the task than the experimental group in the test session. The difference was only significant for the latency from the first to the second ball push.

3.2.3 Impact of ACC Inhibition on Perseveration

In some cases, the animals repeatedly pushed the second ball after receiving a reward, and this was interpreted as perseverative behavior. The excessive pushes of the second ball were not registered in the raw data output and was therefore manually counted from experiment videos of the learning session.

A high variability in the number of perseverative ball pushes was seen in both groups, but the variability was markedly higher in the experimental group (experimental group: *M* = 88.75, *SD* = 41.36, control group: *M* = 80.25, *SD* = 11.53) (fig. 3.5b). A Mann-Whitney U

test revealed no differences in perseverative behavior across the two groups (U = 5.0, p = .486), indicating that the temporary inhibition of the ACC did not have any impact on perseveration.

3.2.4 Difference Between the Learning and the Test Session

As described in paragraph 2.5.3, we did an additional test session of both groups, in which the experimental animals did not have any manipulations of neurons in the ACC. This was done in case the inhibition of the neurons in the ACC would induce unexpected reactions which could interfere with the animal's ability to exhibit learning. It was quickly apparent that the experimental animals did not display any of these unexpected side effects which this additional test session was designed to uncover. However, the experimental animals unexpectedly showed a significantly lower accuracy than the control group in the test session.

This reduced accuracy when neurons in the ACC were not inhibited prompted further analyses. A Wilcoxon Signed Rank test was conducted to compare the accuracy from the learning and the test session for each group, respectively. This test revealed a non-significant trend towards reduced accuracy in the test session for the experimental group (Z = -1.826, p = .068). The same test conducted for the control group showed no such trend (Z = -0.365, p = .715).

Comparison of the latency from the first to the second ball push in the learning session and the test session revealed no difference for the experimental group (Z = -0.365, p = .715), nor were there any trend towards an increased latency in the test session. There was similarly no difference in latency from the first to the second ball push in the control group (Z = -0.000, p = 1).

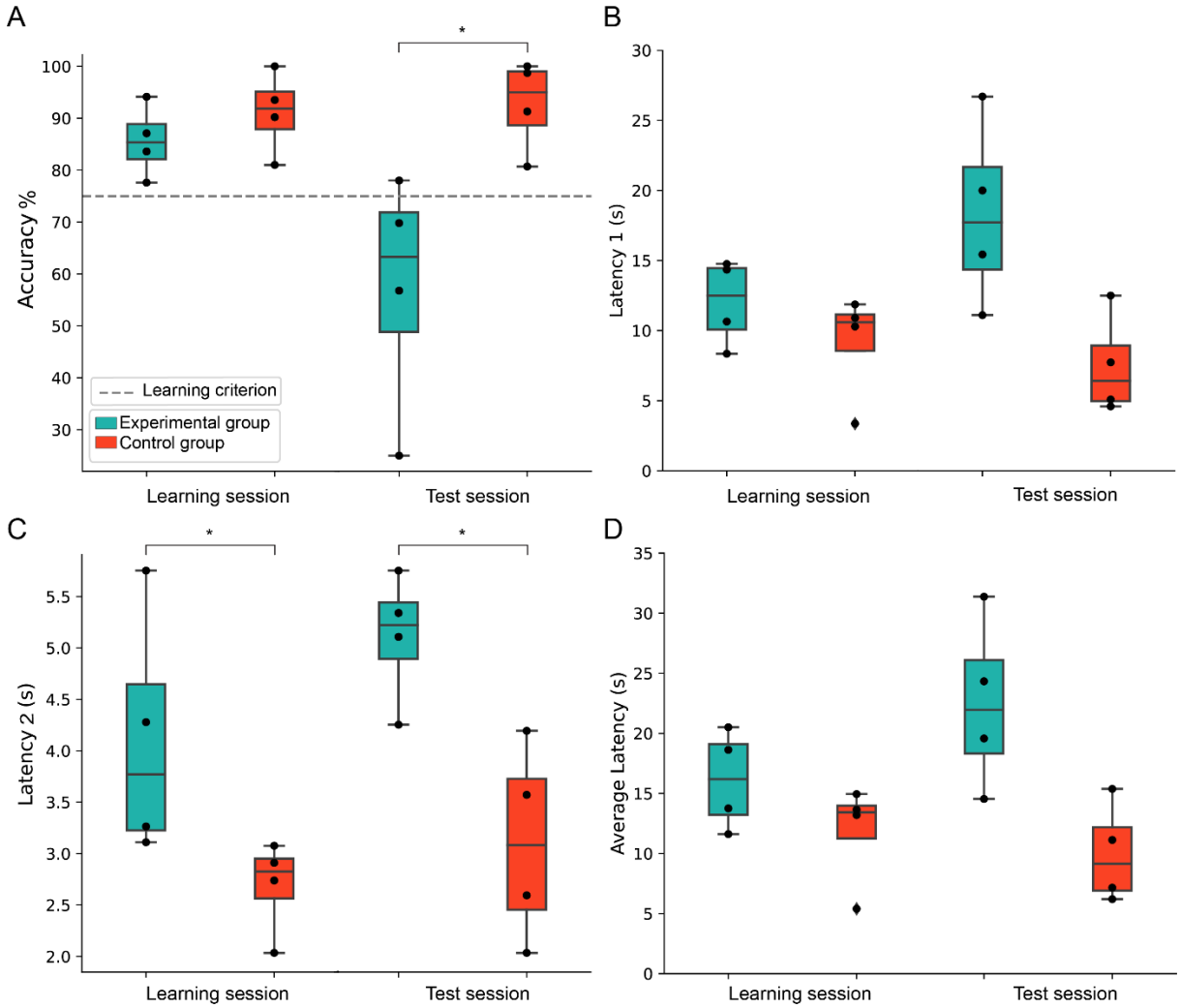

Figure 3.4. Impact of inhibition of neurons in the ACC on accuracy and performance speed. A) The accuracy of the different groups in both the learning and the test session. The experiment group had a significantly lower accuracy than the control group in the test session. **B)** The latency from when the first ball lit up until it was pushed did not differ significantly between the groups. **C)** The experimental animals had significantly increased latency from the first ball to the second ball was pushed, both in the learning and in the test session. There were no significant differences between the learning and the test session in either group. **D)** The average latency of a whole completed trial, combining the two previous measures, did not differ significantly neither between the groups nor between sessions. * $p < 0.05$

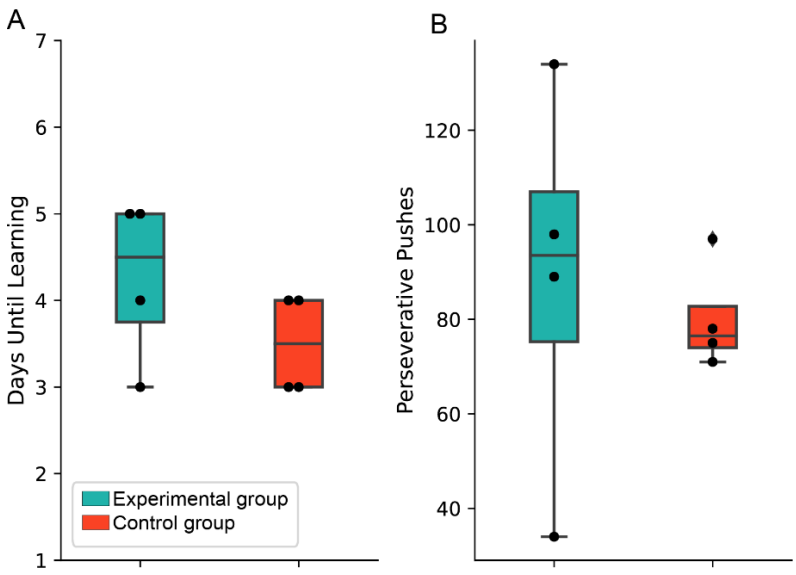

Figure 3.5. Impact of inhibition of neurons in the ACC on number of sessions needed to reach the learning criterion and perseveration. A) The groups did not differ in the speed in which the animals learned the task, seen as number of sessions needed to reach the learning criterion. **B)** The groups did not differ significantly in number of perseverative pushes of the balls.

4. DISCUSSION

The aim of the current project was to investigate how temporary inhibition of excitatory neurons in the ACC would impact reward-driven, non-social associative learning in rats. This was done as part of a larger series of experiments for a PhD project investigating the role of ACC in rats during observational, reward-driven learning. To this end, we trained rats to perform a sequential sensory-motor task, consisting of pushing two ping-pong balls in a specific order. In one condition, we indirectly inhibited excitatory neurons in the ACC through optogenetic stimulation of GABAergic interneurons expressing the opsin Channelrhodopsin-2 (Chr2), which in turn inhibited surrounding excitatory neurons during the training sessions for the animals. The rats were trained using intermittent rewarding electrical stimulations to the medial forebrain bundle (MFB) through a chronically implanted bipolar stimulating electrode. The goal was to have the rats perform the task sequence correctly within a 30 second limit for each ball push, receiving a reward only after the second ball push, and to reach a success rate of >75% correct trials of all possible trials during a 30-minute session, within seven training sessions. A control group without optogenetic manipulation of neurons in the ACC was used as comparison for their performance and the effect of the inhibition. We found that manipulation of neurons in the ACC did not impact the rats' ability to learn, nor did it impact their accuracy on the task, but it did increase the time needed to perform the task. Moreover, we saw a decrease in accuracy in the group with optogenetic manipulation of neurons in the ACC compared to controls when tested in a later session without manipulation of the ACC neurons.

4.1 ACC Inhibition does not Eliminate Learning

The results from the current experiment indicate that the manipulation of neurons in the ACC did not eliminate the rat's ability to learn the task. In fact, all the animals in the experimental group were able to learn the task within the limit of seven sessions, in contrast to only two-thirds in the control group, which revealed a trend towards the experimental animals learning the task better than the control group. Given that all the control animals that did learn the task did so after only three-four sessions, it can be speculated whether the lack of learning in these two control animals were just a matter of chance, and a larger sample size is needed to draw conclusions. Nevertheless, these results clearly indicate that the temporary

inhibition of excitatory neurons in the rat ACC did not disrupt the ability to learn a sensory-motor task driven by reward.

In addition to the finding that manipulation of neurons in the ACC did not disrupt the overall ability to learn the task, we did not find any support for the hypothesis that the manipulation would increase the number of sessions needed to learn the task. With the current dataset, the experimental animals needed more sessions than controls to learn the task on average, but to a small and non-significant effect. The fact that the two non-learners in the control group were excluded from this analysis further refutes this hypothesis, as inclusion of them would have increased the mean number of sessions needed to learn the task for the control group.

Previous studies investigating ACC function have found that it contributes to a wide array of cognitive functions that can be associated with learning in different circumstances. Lesioning or temporarily inhibiting the rodent ACC has been found to impact the animals' ability to form stimulus-reinforcer associations (e. g. Bussey et al., 1997a; 1997b), their ability to correct their behavior after performing errors (Narayanan & Laubach, 2008) and to reduce performance on attentional behavioral tasks (e. g. Fisher et al., 2020; Wu et al., 2017). As the current experiment was meant as a control experiment in a paradigm investigating observational learning, there were some constraints as to which cognitive processes the experiment was able to measure. First, the task itself is not validated for the use of measuring specific cognitive functions, and direct comparisons to other studies are difficult as we used a novel study design. Additionally, as we used shaping with rewards at intermittent steps, a completed trial with rewards at multiple steps could not be considered a successful trial. Only the later sessions without manual stimulations given by the researchers were suitable for comparisons, which made it difficult to measure the gradual learning process from session to session in the earlier stages.

Our findings that the animals with optogenetic manipulation of the neurons in the ACC did not show an impaired ability to learn the task is contrary to the studies indicating that the ACC is involved in the early stages of learning (e. g. Bussey et al. 1997a; Gabriel, 1990; Parkinson et al. 2000). However, Bussey et al. (1996) did find that when the animals had learned to associate a lever press with reward prior to lesioning and the cognitive load was low, using only one single discriminative rule, the ACC lesioned animals learned the discriminative rule faster than controls. They speculated whether this was due to two

competing systems, one for stimulus-reinforcer associations and one for stimulus-response associations, with the former being mediated by the ACC. They argued that lesioning the ACC could lead to less competition for the stimulus-response system, as a result of a compromised stimulus-reinforcer system. In the current experiment, the animals did not have any previous associations of what could result in a reward, unlike the animals in Bussey et al. (1996). This absence of prior reward associations could be expected to impair learning if the idea of a stimulus-reward system in the ACC is correct. However, there are differences in reinforcement strategy between the current study and Bussey et al. (1996) which could have changed the reliance on ACC on forming representations of rewards necessary to complete the task. In Bussey et al. (1996), the animals needed to perform a single action, a lever press, and then collect a sucrose pellet in a magazine. In the initial parts of the current experiment, the animals were rewarded, with temporal precision, just for moving towards a ball by chance. In other words, the mental representation of a reward was present long before they learned the entire sequence, as they initially mainly moved towards "what felt good". While direct comparisons are difficult, it can be argued that the animals in our experiment did not need to form the stimulus-reinforcer associations Bussey et al. (1996) referred to. Instead, as the animals were rewarded at intermittent steps, they may only have needed to learn the stimulus-response association that resulted in reward, which according to Bussey et al. (1996) was facilitated by ACC lesions.

Bussey et al. (1997a) found that ACC lesioned animals were impaired on an extinction task, with a reduced ability compared to controls to omit lever press responses when no reinforcers were given. This impaired extinction learning might have been advantageous in the current experiment. During the early stages of the experiment, while the animals in our experiment gradually came to understand that ball pushes resulted in rewards, they were rewarded for pushes to both balls, imitating the whole correct sequence but with additional rewards from pushes to the first ball. If the inhibition of neurons in the ACC resulted in impaired extinction learning, this might have increased the animals' tendency to continue to push the first ball in absence of a reward. This could make them perform the whole task correctly instead of ceasing to hit the first ball during the last stages of training when reward for that action was removed. Taken together, the nature of our paradigm could have facilitated learning during inhibition of neurons in the ACC by using shaping as a reinforcing strategy, if the hypotheses of ACC's involvement in stimulus-reinforcer associations and extinction learning is correct. This could in turn could account for why we

did not see the expected impairment in the early stages of learning as a result of manipulating neurons in the ACC.

4.2 ACC Inhibition does not Impact Accuracy but does Impact Performance Speed

Although the mean accuracy for the experimental group from the learning session was lower than for the control group, the difference was small and non-significant. Thus, we did not find support for the hypothesis that inhibiting neurons in the ACC would lead to lower accuracy on the task. This contrasts with previous studies using attentional tasks like the 3-CSRTT, the 5-CSRTT and rodent CPT's, in which ACC inhibition led to reduced accuracy (Chudasama et al., 2003; Fisher et al., 2020; Passetti et al., 2002; Wu et al., 2017). It might be due to the different experimental conditions. In the mentioned experiments, the animals were pre-trained on the task before lesioning, and the time window for responding after stimulus presentation was short, typically around 5 seconds. In the current experiment, the animals had to push each ball within 30 seconds from the light turned on. This large time window for responses could explain the absence of differences in accuracy between the experimental and control group. The experimental animals might have decreased their accuracy more with a shorter time window for responding. The fact that the experimental group did spend significantly longer time than the control group from the first to the second ball push, which is seen as the most informative measure of performance speed in this task, supports this view. This increased response latency is in accordance with the hypothesis that inhibition of neurons in the ACC would increase the time spent on performing the task, and with previous studies (e. g. Koike et al. 2016; Passetti et al. 2002).

The increase in response latency might be seen as an attentional impairment, in agreement with the suggestion that ACC is involved in attention. However, the task used in the current experiment is not validated as a measure of attention such as the 5-CSRTT. Consequently, while the results of the current experiment are in agreement with previous attentional experiments that found increased response latencies, the claim that this is due to attentional impairments in this particular experiment remains speculative. For a more suitable comparison to previously used paradigms (e. g. Fisher et al., 2020; Hvoslef-Eide et al., 2018; Koike et al., 2016; Passetti et al., 2002), it would be interesting to test if a pre-trained animal, trained without inhibition of neurons in the ACC, would increase their response latency and reduce their accuracy if tested with the inhibition of neurons in the ACC and with a shorter response time window.

Alternatively, the increased latency might have been due to reduced overall locomotion following inhibition of neurons in the ACC. This is however unlikely, given that previous research mostly found no difference in locomotion following ACC lesions (Cardinal et al., 2002; Koike et al., 2016; Narayanan et al., 2006; Wu et al., 2017), with some finding increased locomotion (Rudebeck et al., 2006). Additionally, the difference between the groups on the latency from when the first ball lit up until it was pushed was not significant, which might be expected if the experimental animals decreased their overall locomotion. Attempts were made to control for locomotion in the current experiment by using different animal tracking software packages on the videos from the experiment sessions and get an overall measure of animal movement in a session. However, the software packages tested were not able to reliably track the movement of the animals. This was due to the videos capturing animal behavior not being sufficiently bright and the floor in the experimental box being black, not giving enough contrast for the tracking software to reliably distinguish the animal from its surrounding. Consequently, we were not able to control for locomotion within the time frame of this project.

4.3 Impact of ACC Inhibition on Other Suggested Functions of the ACC

4.3.1 Perseveration & Inhibition

We did not find any indications of an impact of inhibition of neurons in the ACC on the number of perseverative responses, measured as repeated pushes to the second ball after receiving a reward. This is not surprising, as previous indications of perseverative behaviors following ACC lesions have been vague at best. Chudasama et al. (2003) found increased perseverative behavior when the inter-trial intervals were shorter than in the baseline condition and were of variable lengths, but not in other conditions. Passetti et al (2002) distinguished between the cingulate cortex (Cg) and the prelimbic (PL) and infralimbic cortex (IL), and found increased perseverative behaviors following combined lesions to the PL and IL, and when lesioning all the three areas, but not after lesions only to the cingulate cortex. Similarly, Fisher et al. (2020) found that PL lesions led to increased responses during time-outs, but they did not distinguish between premature and perseverative responses. As the PL and IL are included in some definitions of the ACC, we wanted to explore perseverative behavior in our experiment. However, we targeted the Cg1 and Cg2, not the PL and IL, and did therefore not formulate a specific hypothesis for perseverative behaviors following inhibition of neurons in the ACC.

On the other hand, there have been multiple studies finding increased premature responses following ACC lesions (e. g. Hvoslef-Eide et al., 2018; Muir et al., 1996; Narayanan et al., 2006; Wu et al., 2017), which is seen as disinhibition. As described in the result section, the animals in the current experiment did not usually display behavior that would indicate an understanding of the light cues even when they got above 75% correct trials in a session. This made it difficult to differentiate premature responses from just a lack of understanding, and the paradigm used here was therefore unsuitable for investigating inhibition. In order to do so, we would need to train the animals for longer periods to ensure that they understood the light cues. Alternatively, the current experiment could have implemented a "punishment" for premature responses, which has been used in previous studies which measures inhibition (e. g. Hvoslef-Eide et al., 2018; Muir et al., 1996; Narayanan et al., 2006), in the form of a time-out during which no rewards could be obtained. This way, premature responses would have been more informative of disinhibition than what we could currently measure, and it would have been easier to compare with other studies in which a lack of inhibition following ACC lesions is a consistent finding.

4.4.1 Cost-Benefit Analyses and Reward Estimation

In the current experiment, we could not assess the animal's cost-benefit analysis or reward estimation directly. Studies on reward estimation and cost-benefit analyses (Rudebeck et al., 2006; Schweimer & Hauber, 2005; Walton et al., 2003) have found an altered willingness to invest effort for rewards, where rats in which the ACC was lesioned show a preference for a low-effort but low-reward option over a high-effort and high-reward option. In our paradigm, there were no option for different reward sizes, but the animals were clearly willing to invest efforts to obtain rewards. Thus, the impact of the role of the ACC in reward estimation may only be apparent when there are different options in place, not when the option is between a reward and no reward. In other experiments on the ACC using rewards as a reinforcer for certain behaviors, such as the ones on discriminative learning and attention (e. g. Bussey et al., 1996;1997a;1997b; Chudasama et al., 2003; Koike et al., 2016), there are similarly no considerations of reward estimation alterations. The animals in these studies are still sufficiently motivated by the rewards to perform the different tasks.

If the animals in our experiment with inhibition of neurons in the ACC had not displayed learning, we could not have clearly distinguished whether this was a result of for instance impaired attention or due to an altered willingness to invest effort for rewards. As all

the experimental animals did display learning and the motivation needed to obtain rewards, we can conclude that the alterations in cost-benefit analyses seen in other studies were, if present in the current experiment, not sufficient to disrupt the motivation for learning. However, we cannot exclude the possibility that the increased latency from the first to the second ball hit was a result of lack of motivation as much as an attentional impairment.

4.4.2 Long-Term Memory

Previous studies have found that the ACC is involved in long-term memory, for instance in memories for objects (e. g. Pezze et al. 2017). If the animals in the current study had not been able to encode or retrieve memories of the task and the arena, it would likely have impaired their learning from session to session. Nevertheless, our experimental animals did display memory for the task, as they did not need more sessions than the control group before they reached the learning criterion. Other studies on ACC function in which the memory for a task is encoded before lesioning or inhibiting the ACC does not seem to display a memory deficit either. For instance, a lever press (e. g. Bussey et al., 1996) or nose pokes in the panels used in a 5-CSRTT (e. g. Chudasama et al., 2003) would require some form a memory of the objects in use for the animal to be able to perform. However, in the studies on long-term memories for objects, there is no direct interaction with the object required. This interaction with an object, whether there be a lever press, a nose poke in a hole in a panel or a push to a ball, might entail a procedural and motoric memory mechanisms which likely rely on more than the ACC. Thus, our results indicate that even if the inhibition of neurons in the ACC lead to disrupted retrieval of memories for objects, it was not sufficient to disrupt the memory retrieval necessary to perform the task.

4.5 State-Dependent Memory

The day after the animals reached the learning criterion, they underwent an additional test session without optogenetic inhibition of neurons in the ACC. The original idea behind this test session was to ensure that the inhibition of neurons in the ACC did not interfere with the animals' ability to display learning, for instance as a result of impaired motor function. It therefore came as a surprise that all the experimental animals performed worse in this session. In fact, only one of the four experimental animals had an accuracy above the learning criterion in this session. While there was no clear difference in accuracy between the two groups in the learning session, the experimental group performed significantly worse than the

control group in the test session. The difference in accuracy for the experimental group between the learning and the test session was not significant, albeit closely, and it can be speculated whether this would have reached significance with a larger sample size.

One possible explanation could be fatigue after training consecutive days. From training animals on the task for the observational learning experiment, it became apparent that some animals decreased their performance if they were trained for multiple consecutive days without a break. This was the reason for having one day without training following the fourth session. If the animals reached the learning criterion in the fourth session, the test session was set to the following day, not giving them a rest day, which could impact their performance. In fact, the two animals in the control group that reached the learning criterion in the fourth session were the only ones to decrease their performance in the test session. This supports the idea of having a rest day after four days in further experiments in the paradigm. However, if fatigue was the only explanation for the decreased accuracy from the learning to the test session, it should have been most apparent in the control group, not in the experiment group. Two of the four animals in the experiment group needed five sessions to reach the learning criterion, giving them a rest day after the fourth session, but all animals in the experiment group decreased their accuracy in the test session. In contrast, none of the learners in the control group got a rest day, as all reached the learning criterion within four sessions, and only half reduced their accuracy in the test session.

Another possible explanation for the reduced accuracy in the test session is what is termed state-dependent memory. State-dependent memory is the tendency for memories to be more easily retrieved in a similar or the same mental state to when the memory was encoded, whether that be an emotional, physical or drug-induced state (see Zarrindast & Khakpai, 2020 and Radulovic et al., 2018 for reviews). For example, researchers found that when injecting the GABA receptor agonist Gaboxadol into the hippocampus in mice either before fear conditioning or before memory retrieval following fear conditioning, the mice reduced their fear expression, indicating impaired learning and memory retrieval, respectively. However, when the mice were injected with Gaboxadol in the hippocampus both before encoding and retrieval, creating the same “state” in both conditions, their freezing responses did not differ from controls (Jovasevic et al., 2015). In our experiments, by temporarily manipulating neurons in the ACC by exciting interneurons, and thus changing the excitatory/inhibitory balance, we could have artificially created a specific mental state in the animals in which they were able to learn the task sequence. When tested without this inhibition of excitatory

neurons in the ACC, the performance of the animals might have been impaired as the animal was in a different "state" than during the previous sessions, similar to the findings by Jovasevic et al. (2015).

4.6 Methodological Limitations

4.6.1 Experimenter Effect and Potential Biases

A potential limitation in the current study were inconsistencies that having multiple experimenters can bring. Four of the animals were trained by the author, two control animals were trained by my colleague Devika Kurup, and one experimental and three control animals were trained by my co-supervisor Ida V. Rautio. Laboratory animals have been found to react differently to different handlers (Chesler et al., 2002). To counter this issue, the animals were socialized by the experimenter performing the experiment, as familiarity to the experimenter can increase the consistency in testing (Driel & Talling, 2005). Additionally, having different experimenters can lead to inconsistencies in judgement of behavior, termed experimenter effect, which has been found to sometimes produce markedly different results (Bohlen et al., 2014). Our training protocol was written to be intuitive and specific, reducing variability in our training strategy. Nevertheless, some of the training was dependent upon the experimenter's judgement. For example, what was considered an "approach to a lit ball" (what would result in rewarding stimulation on step 2 of the training protocol), may have varied across experimenters. In fact, the experiments with the only two animals that did not reach the learning criterion were both performed by the same experimenter, which may point towards an experimenter effect.

Additionally, the experimenters were not blinded to which experimental condition the animals were in. This can lead to performance bias, an intentional or unintentional difference in how the animals in the two experimental conditions were treated (e. g. O'Connor & Sargeant, 2014). Due to the fiber-optic cannula implants it was not possible for the experimenters to be blind to the experimental conditions of the animals. As a solution, we could have implanted all the animals with fiber-optic cannulas and performed a sham injection on the control animals, by such making the groups only differ in terms of opsins. This would however require more resources, and the experimenter could not perform the surgeries on the animals as this would give away which group the animal belonged to.

4.6.1 Use of Optogenetic Tools

In the experiment on observational learning on which this project was based, there was a need for inhibiting the neurons in the ACC with temporal precision. Chr2 targeted at GABAergic interneurons was chosen, as it enables effective manipulation which is highly temporally precise (Yizhar et al., 2011a). The efficacy of the virus used was tested by Ida V. Rautio and showed an efficient and long-lasting inhibitory effect. As the current experiment was meant as a control experiment in this existing paradigm, it was considered necessary for the sake of consistency and comparability to inhibit neurons in the ACC using the same methodology in the current experiment.

Many of the previous experiments on ACC function used lesions as a method of disrupting neuronal activity (e. g. Fisher et al., 2020; Hvoslef-Eide et al., 2019; Schweimer & Hauber, 2005). By using optogenetic tools and temporary manipulation of neurons, one can avoid the drawbacks of permanent interventions such as compensatory processes following lesions, in which non-damaged neurons in the same or connected brain areas can compensate for the neuronal loss and the animal regains some brain functions over time (Vaidya et al., 2019). The current experiment could last more than a week, in addition to a minimum of six days healing period following surgery. Lesioning the animals could therefore have led to the animals regaining some ACC function before the experiment ended. Lesions would also have excluded the possibility for testing the animals without inhibition of neurons in the ACC after they reached the learning criterion. However, while there are drawbacks of using lesions, other tools outside of optogenetics using Chr2 exists. If the current experiment was not constrained to use the same technology as the observational learning experiment, other options could have been considered.

In the current experiment, we needed the neurons in the ACC to be inhibited for a full 30-minute session. The use of Chr2 for long lasting manipulation is uncommon, and the author has not succeeded in finding any other studies using optogenetic inhibition with Chr2 for such prolonged periods. The stimulation protocol was tested and verified by my co-supervisor Ida V. Rautio with the use of Neuropixels probes to record neural activity while indirectly inhibiting the excitatory neurons in the ACC. The first two animals tested showed pronounced tissue damage following the prolonged light exposure, as they were tested with continuous and not pulsating light (see Appendix B). Ultimately, we found a light intensity

and pulsating frequency which did functionally inhibit excitatory neurons in the ACC for a full 30-minute session without damaging the brain tissue.

In addition to Chr2 not being commonly used for such prolonged time periods, the usage of fiber-optic cannula implants also presents a problem of determining how far the light has spread in the tissue and thus how much tissue is affected. In the animals used for developing the optogenetic protocol which were Neuropixels probes, the effective spread could be calculated. However, we cannot determine the effective spread of the light precisely in each experimental animal without neural recordings, which was not a part of this study.

Alternatively, other chemo- or optogenetic tools validated for prolonged neuronal manipulation could have been used to inhibit principal neurons in the ACC for this study. For instance, step-function opsins (SFOs) can activate neurons for as long as 30 minutes with a single light pulse (Guru et al., 2017; Yizhar et al., 2011b), which would have been suitable for our experiment as it reduces the probability of tissue damage. Alternatively, we could have used a Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based tool, as used by Koike et al. (2016), which effects last for an hour or more (Roth, 2016). By using DREADDS, we would also have avoided the need for fiber-optic cannula implants, as the neurons can be activated through intraperitoneal injections of clozapine-N-oxide (CNO). Central administration of the drug could ensure manipulation of all neurons expressing the chosen DREADD, which additionally could enable manipulation of a larger area of the ACC, which in turn could have been identified using immunohistochemical staining. On the other hand, injections before every experiment session could influence the animals' behaviors due to stress, which we avoided by using optogenetics. Thus, while it was in the interest of the project to use of Chr2 for temporary inhibition of neurons in the ACC, subsequent studies should consider using other chemo- or optogenetic tools instead, for instance SFOs or DREADDs.

4.7 Conclusion

The aim of this thesis was to investigate the involvement of ACC in reward-driven, associative learning in rats, to provide a counterpart for a larger series of experiment for a PhD project investigating ACC-dependent, reward-driven social learning. The results from the experiments clearly indicated that the temporary inhibition of neurons in the ACC did not disrupt the animal's ability to learn a sequential tapping task. It did, however, increase the time used to perform the task. Additionally, we found indications consistent with state-

dependent learning, manifested as reduced performance of rats trained with temporary inhibition of the ACC when tested on the task without neurons in the ACC inhibited. These results will make it easier to isolate the social learning aspects relevant for the observational learning experiments, by demonstrating that inhibition of principal neurons in the ACC does not disrupt the non-social learning version of the ball-pushing task. By itself, this project is a valuable addition to the complex literature on the ACC, showing that an animal can learn a non-intuitive sensory-motor task through manual shaping while neurons in the ACC are being inhibited.

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Appendix A: Supplementary Methods

Supplementary Table 1. List of Animals Used in Experiment

ID number, date of birth (DOB), stimulation strength in mV, date of viral injection (DOV), date of implantation (DOI), initials of surgeon (Ella Holt Holmberg; EHH, Devika Kurup; DK, Ida V. Rautio; IVR), date of death (DOD), group (control: C, experimental: E).

Animal ID	Strength	DOB	DOV	DOI	Surgeon	DOD	Group
26887	24-26	20/08/20	-	16/11/20	EHH	30/12/20	C
26991	24-26	18/09/20	04/12/20	30/12/20	IVR	10/01/21	E
27037	26	07/10/20	11/12/20	10/01/21	EHH	28/01/21	E
27040	34	07/10/21	17/12/20	14/01/21	EHH	29/01/21	E
27075	26	13/10/20	-	17/02/21	DK	08/04/21	C
27077	25	13/10/20	-	27/01/21	DK	05/03/21	C
27326	26-27	21/03/21	-	15/06/21	IVR	03/07/21	C
27399	24-26	16/04/21	24/06/21	21/07/21	IVR	01/08/21	E
27400	26-30	16/04/21	-	19/07/21	IVR	05/08/21	C
27401	26-30	16/04/21	-	22/07/21	IVR	05/08/21	C

Supplementary Table 2. List of Animals Used for Surgery Practice/Non-Working Implants/Complications During Experiment

*ID number, date of birth (DOB), date of viral injection (DOV), date of implantation (DOI), initials of surgeon (Ella Holt Holmberg; EHH, Devika Kurup; DK, Ida V. Rautio; IVR), date of death (DOD). * Exposed to light stimulation in the ACC to test for tissue damage*

Animal ID	DOB	DOV	DOI	Surgeon	DOD
26806	17/07/20	-	26/10/21	EHH	02/11/20
26849	17/07/20	-	08/10/20	EHH	17/10/20
26860	12/08/20	-	15/11/20	EHH	23/11/20
26861	12/08/020	-	09/11/20	EHH	16/11/20
26862	12/08/20	-	06/11/20	EHH	06/11/20
26993*	08/09/20	10/12/20	03/01/21	EHH	12/01/21
27038*	07/10/20	14/12/20	07/01/21	EHH	22/01/21
27039	07/10/20	16/12/20	11/01/21	EHH	22/01/21
27135	02/11/20	-	21/01/21	EHH	03/02/21
27136	02/11/20		02/02/21	EHH	15/02/21
27137	02/11/20		09/02/21	EHH	18/02/21

Supplementary Table 3. List of Animals Used for the Development of the Optogenetic Protocol
ID number, date of birth (DOB), date of viral injection (DOV), date of implantation (DOI), initials of surgeon (Ella Holt Holmberg; EHH, Devika Kurup; DK, Ida V. Rautio; IVR), date of death (DOD).

Animal ID	DOB	DOV	DOI	Surgeon	DOD
26804	17/07/20	22/09/20	22/10/20	IVR	24/10/20
26805	17/07/20	21/09/20	30/11/20	IVR	01/12/20
26735	15/06/20	18/09/20	05/10/20	IVR	02/11/20
26992	18/09/20	03/12/20	27/12/20	IVR	30/12/20

Protocol for MFB Implantation and Fiber-Optic Cannulas Implantation
** Only for implantations of fiber-optic cannulas*

Preparation the day before surgery

Fill isoflurane

Autoclave tools needed

Prepare two syringes with saline and one with hydrogen peroxide and keep it in the fridge. Put necessary equipment on surgery table: Surgical drape, Sugi, sutures, scalpel blade, klorhexidin, dental cement, three syringe needles, one needle for cutting dura

Fill a bottle with 70% ethanol

Find a usable stimulating electrode and leave it on the table

Preparation on the day of surgery

Weight the animal and prepare weight-appropriate medication dosages

Put cotton swabs in UV-cabinet and turn on the UV light

Turn on the heating plate and cover it with a fresh towel

Take the toolbox out of the autoclave and fill it with 70% ethanol

Open and unfold the surgical drape and leave the tools needed in the beginning of the surgery on it for the ethanol to evaporate

Get the Kwik-Sil and syringes with saline and hydrogen peroxide

Fill a small cup of ethanol (two if implantation of fiber-optic cannula*)

Tightly fasten the stimulating electrode on an alligator clamp on a stereotaxic arm and lower it down so the whole electrode is covered in ethanol

Tightly fasten the fiber-optic cannulas on an alligator clamp on a stereotaxic arm and lower it down so the whole electrode is covered in ethanol*

Get the box with cotton swabs and leave it on the surgery table

Fill Styrofoam box with ice and place one syringe with saline in it

Ensure the mouthpiece is set to 9 degrees

Spray the whole surgery area with 70% ethanol

Anesthesia

Set oxygen flow to 0.5-0.6 l and isoflurane flow to 0.5-0.6

Fill chamber with 5% isoflurane for minimum 10 minutes

Place the rat in the chamber

Adjust tubes so the airflow is going to the mouthpiece

When sedated, bring the animal out and quickly shave the head above a bin

Place the animal on the heating plate and place its snout in the mouthpiece

Lower the oxygen flow to 0.3-0.4 and isoflurane flow to 0.3-0.4 and concentration to 1.5-3%

Prepare animal

Tightly fix the head with ear bars, ensure that the head is level

Lower the heating plate to ensure that the rat has free airways

Apply eye ointment

Shave off remaining fur on the head

Inject analgesics subcutaneously on the back and local anesthesia to the shaved area

Inject saline, maximum 2.5 mL at each injection site

Cover the shaved area with chlorhexidine, remove any remains of fur and dirt

Cut nails

Make a straight incision, ensure that both bregma and lambda are visible

Wipe off excess blood and slime with cotton swabs

Attach four hemeostatic clamps to the tissue and tape them on the stereotaxic frame to ensure visibility

Scrape the scalpel blade on the skull to create a rough surface for the dental cement to attach to

Apply hydrogen peroxide, wait a few seconds and then remove it with Sugi and apply saline

Use the microscissors to remove any damaged tissue

Repeat the process with hydrogen peroxide and saline until no damaged tissue is present and end with one round of hydrogen peroxide and saline

Craniotomy and implantation

Mark bregma with a small cavity with the burr

From bregma, move the drill to the cite of the craniotomy to the electrode implantation: AP: -2.8, ML: +1.7

Drill through skull while continuously applying the cold saline with a syringe to avoid heating the bone

Slightly move the drill around to create a larger craniotomy for visibility

Open the craniotomy from the viral injection *

Drill five craniotomies for anchor screws, one to the right of the midline, two to the left and two behind lambda

Bend a syringe needle and use it to cut dura in the craniotomy hole

Cut dura in the viral craniotomy*

Screw in the anchor screws, 2 ½ turns

Implantation

Get the stereotaxic arm with the alligator clamp with the fiber-optic cannulas implant tightly fixed*

Adjust the stereotaxic arm to 20 degrees*

From bregma, move the fiber-optic cannulas to the craniotomy: AP: +3.1, M: 0*

Ensure that the fiber-optic cannulas are parallel to each other*

Slowly lower the fiber-optic cannulas to DV: 2.1*

Get the stereotaxic arm with the alligator clamp with the stimulating electrode fixed

Quickly rinse the electrode with saline from a syringe

Attach the stereotaxic arm to the stereotaxic frame

Make sure that the electrode is straight and perpendicular using edges on the stereotaxic frame for reference. Use forceps to straighten it if needed

From bregma, move the electrode to the same coordinates as the craniotomy

Slowly lower the electrode into the brain, DV: 8

Mix Kwik-Sil on a piece of parafilm with a Sugi

Carefully apply Kwik-Sil to the craniotomy, to the electrode and to the fiber-optic cannulas*, ensuring to never directly touch the electrode

Mix Super-Bond in the designated mixer bowl

Apply Super-Bond to the skulls, screws and Kwik-Sil on the electrode

Apply Super-Bond to the fiber-optic cannulas*

Mix the dental cement, with multiple rounds of adding more dental cement liquid

Apply the first layer of dental cement to the skull, screws and electrode

Apply dental cement to the fiber-optic cannulas*

When completely dry, carefully remove the alligator clamp holding the electrode

Carefully remover the alligator clamp holder the fiber-optic cannulas*

Apply an additional layer of cement

When dry, search for sharp cement edges with cotton swabs and drill them off if needed

Remove any residues of dead tissue or cement

Remove the hemeostatic clamps and use the microscissors to remove the tissue it has been attached to if it is damaged

If there is any open space around the implant, suture the wound in front to close it

Inject remaining saline

Protocol for Viral Injections to the ACC

Preparation the day before surgery

Fill isoflurane

Autoclave tools needed

Prepare two syringes with saline and one with hydrogen peroxide and keep it in the fridge. Put necessary equipment on surgery table: Surgical drape, Sugi, sutures, scalpel blade, klorhexidin, dental cement, three syringe needles, one needle for cutting dura

Fill a bottle with 70% ethanol

Find a usable stimulating electrode and leave it on the table

Preparation on the day of surgery

Get the viral vector from the freezer and slowly defrost it in a Styrofoam box with ice

Add a tiny drop of Fast Blue to the solution and quickly mix it in the Eppendorf tube using a centrifuge

Get the pre-pulled pulled glass microcapillary pipette

Use a Hamilton Syringe to extract the mixed viral vector and then use the syringe to fill the microcapillary pipette with a small dose of viral vector

Use the Hamilton Syringe with vegetable oil and fill the microcapillary pipette with oil above the viral vector. Ensure there are no air bubbles.

Leave the microcapillary pipette with the viral vector in the fridge

Put cotton swabs in UV-cabinet and turn on the UV light

Turn on the heating plate and cover it with a fresh towel

Take the toolbox out of the autoclave and fill it with 70% ethanol

Open and unfold the surgical drape and leave the tools needed in the beginning of the surgery on it for the ethanol to evaporate

Get the Kwik-Sil and syringes with saline and hydrogen peroxide

Get the cotton swabs and leave them on the surgery table

Fill Styrofoam box with ice and place one syringe with saline in it

Ensure the mouthpiece is set to 9 degrees

Spray the whole surgery area with 70% ethanol

Anesthesia

Set oxygen flow to 0.5-0.6 l and isoflurane flow to 0.5-0.6

Fill chamber with 5% isoflurane for minimum 10 minutes

Place the rat in the chamber

Adjust tubes so the airflow is going to the mouthpiece

When sedated, bring the animal out and quickly shave the head above a bin

Place the animal on the heating plate and place its snout in the mouthpiece

Lower the oxygen flow to 0.3-0.4 and isoflurane flow to 0.3-0.4 and concentration to 1.5-3%

Prepare animal

Tightly fix the head with ear bars, ensure that it is straight

Lower the heating plate to ensure that the rat has free airways

Apply eye ointment

Shave off remaining fur on the head

Inject analgesics subcutaneously on the back and local anesthesia to the shaved area

Inject saline, maximum 2.5 mL at each injection site

Cover the shaved area with chlorhexidine, remove any remains of fur and dirt

Cut nails

Make a straight incision, ensure that there is enough room in front of bregma

Wipe off excess blood and slime with cotton swabs

Attach four hemeostatic clamps to the tissue and tape them on the stereotaxic frame to ensure visibility

Apply hydrogen peroxide, remove it with Sugi and apply saline

Use the microscissors to remove any damaged tissue

Repeat the process with hydrogen peroxide and saline until no damaged tissue is present and end with one round of hydrogen peroxide and saline

Craniotomy and injection

Mark bregma with a small cavity with the burr

From bregma, move the drill to the cite of the craniotomy of the viral injection: AP: +2.5, ML: +: ±0,5

Drill through skull while continuously applying the cold saline with a syringe

Slightly move the drill around to create a larger craniotomy for visibility

Remove any skull residues

Bend a needle and use it to cut dura

Get the microcapillary from the fridge and slowly insert the needle on the holder that is connected to the controller, ensuring no bubbles

Attach the microcapillary pipette holder to the stereotaxic frame

From the small cavity on bregma, move the microcapillary pipette to the coordinates of the craniotomy

Use a scissor and cut off the tip of the microcapillary pipette

Start the microinjector pump with 50 nL/min speed and eject a small drop of viral vector to ensure that the pump works

Slowly lower the microcapillary pipette into the brain

Initiate the injection while lowering the microcapillary pipette

Stop at DV: +2

When 500-700 nL is injected, wait for 10 minutes the microcapillary pipette to avoid backflow

Slowly retract the microcapillary pipette

Repeat the process in the other hemisphere

Leave everything that has been in contact with the viral vector in a brain cup with Virkon

Cover the craniotomy with Kwik-Sil

Suture the incision, ensure no air is left underneath the skin

Inject remaining saline

Supplementary Table 4. Chemicals

Bovine Serum Albumin (BSA)	Merck KGaA	Germany
Cresyl Violet 0.5 g Cresyl Violet Acetate	Merck KGaA	Germany
Dimethyl Sulfoxide (DMSO)	VWR International Ltd.	USA
Entellan	Merck KGaA	Germany
Ethanol 100%	KiiltoClean AS	Norway
Exagon ®, pentobarbital sodium 400 mg/ml	Richter Pharma Ag	Austria
Fast Green FCF	Merck KGaA	Germany
Goat Anti-Rat IgG antibody (Alexa Fluor 546)	Thermo Fisher Scientific	USA
Phosphate buffered saline (PBS) 0.01M	Merck KGaA	Germany
Phosphate buffer (PB) 0.4M	Merck KGaA	Germany
Potassium chloride (KCl)	Merck KGaA	Germany
Paraformaldehyde (PFA)	Merck KGaA	Germany
Red Fluorescent Protein (RFP) antibody 5F8	Chromotek GmbH	Germany
Sodium Bicarbonate (NaCHO3)	Merck KGaA	Germany
Sodium Chloride (NaCl)	VWR International, Ltd.	USA
Sucrose	VWR International, Ltd.	USA

Toluene	VWR International, Ltd.	USA
Tris(hydroxymethyl)aminomethane (Tris)	Merck KGaA	Germany
Triton X 100	Merck KGaA	Germany
Xylene (mixture of isomers)	VWR International, Ltd.	USA

Supplementary Table 5. Materials Used for Surgeries

AAV5-mDIx-Chr2-mCherry-Fishell-3 Viral Vector	Kavli Viral Vector Core Facility	Norway
Anesthetic gas vaporizer	MSS International	UK
Bipolar Stimulating Electrode	Plastics One	Canada
Burr 1 mm	Fine Science Tools	USA
Chlorhexidine 5 mg/ml	Fresenius SE & Co. KGaA	Germany
Cotton swabs	Johnson & Johnson	USA
Drill	Kopf Instruments	USA
Ear Bars		
Electric clippers		
Ethanol 70%	VWR International, Ltd.	USA
Fast Blue		
Fiber-optic cannulas, dual	Doric Lenses	Canada
Foreceps		
Heating plate		
Hemostatic clamps		
Hydrogen Peroxide NAF 3%	Norges Apotekerforening	Norway
Isoflo ® vet 100% Isoflurane	Zoetis Inc.	USA
Kwik-Sil ® Silicone Elastomer	World Precision Instruments Inc.	USA
Marcain ®	AstraZeneca AB	UK
Meliodent ® dental cement	Kulzer GmbH	Germany
Metacam ® 2 mg/ml	Boehringer Ingelheim GmbH	Germany
Microcapillary Pipette	World Precision Instruments Inc.	USA

Microinjector pump	World Precision Instruments Inc.	UK
Microscissors		
Parafilm ®	Bemis Company, Inc.	USA
Saline 9 mg/ml	B. Braun Melsungen AG	Germany
Scalpel surgical blade size 22	Swann-Morton Ltd.	UK
Scalpel		
Simplex ®	Optha A/S	Denmark
Spongostan™	Ethicon Inc.	USA
Stereotaxic frame	Kopf Instruments	USA
Sterican ® syringe needle 21 & 25 g	B. Braun Melsungen AG	Germany
Super-Bond ®	Sun Medical Co., Ltd.	Japan
Sugi ® Eyespear pointed tip	Kettenbach GmbH & Co. KG	Germany
Surgical drapes, OP-towel	Barrier Healthcare Ltd.	UK
Sutures 4/0 Supramid ®	Resorba Medical GmbH	Germany
Temgesic ®	Schering-Plough	USA
Virkon	Brage Medical AS	Norway

Supplementary Table 6. Various Materials Used in the Project

Axio Scan Z1	Carl Zeiss AG	Germany
Arduino One	Arduino	
DPSS lasers 150 mV	Shanghai Lasers & Optics Century	China
Fiber-optic Silica Patch Cord 4 m	Doric Lenses	Canada
Hardboard TB4	Thorlabs, Inc.	Germany
Infrared Lights		
Pulse Stimulator Master 9	Microprobes	USA
Microm HM430	Thermo Fisher Scientific	USA
Microtome knife	Nerliens Meszansky AS	Norway
Neuropixels 1.0	Imec	Belgium
Optical Power Meter	Thorlabs, Inc.	Germany

Perfusion Pump	World Precision Instruments Inc.	UK
Polysine ® Slides	Thermo Fisher Scientific	USA
Raspberry Pi	Raspberry Pi Foundation	UK
Raspberry Pi NoIR Camera V2		
Stimulus Isolation Unit ISO-Flex	Microprobes	USA
Superfrost ® Plus	Thermo Fisher Scientific	USA

APPENDIX B: Supplementary Results

Table 1. Individual Data from Learning Session for the Experiment Group

Animal ID	**#26991**	**#27037**	**#27040**	**#27399**
Session number	4	5	5	3
Successful Trials	46	38	54	64
Total Possible Trials	55	49	62	68
Missed Trials	9	11	8	4
Failed Trials	0	0	0	0
Stimulated	46	38	54	64
Proportion of Trials Correct	0.836	0.776	0.871	0.941
Successful Trials per Minute	1.528	1.273	1.813	2.117
Ball 1 Latency	14.35	14.755	10.645	8.352
Ball 2 Latency	4.278	5.752	3.11	3.264
Average Trial Length	18.628	20.507	13.755	11.616
Perseverative pushes	98	89	34	134

Table 2. Individual Data from Test Session for the Experiment Group

Animal ID	**#26991**	**#27037**	**#27040**	**#27399**
Successful Trials	37	25	46	10
Total Possible Trials	53	44	59	40
Missed Trials	16	19	13	30
Failed Trials	0	0	0	0
Stimulated	37	25	46	10
Proportion of Trials Correct	0.698	0.568	0.78	0.25
Successful Trials per Minute	1.238	0.838	1.507	0.33
Ball 1 Latency	15.433	19.999	11.104	26.7
Ball 2 Latency	4.142	4.334	3.438	4.674
Average Trial Length	19.574	24.334	14.542	31.374

Table 3. Individual Data from Learning Session for the Control Group

Animal ID	**#26887**	**#27326**	**#27400**	**#27401**
Session number	3	4	3	4
Successful Trials	55	54	47	82
Total Possible Trials	61	59	58	82
Missed Trials	6	5	11	0
Failed Trials	0	0	0	0
Stimulated	55	54	47	82
Proportion of Trials Correct	0.902	0.915	0.81	1.0
Successful Trials per Minute	1.833	1.798	1.561	2.73
Ball 1 Latency	10.291	10.909	11.868	3.363
Ball 2 Latency	2.91	2.739	3.077	2.035
Average Trial Length	13.201	13.649	14.944	5.398
Perseverative pushes	78	75	97	71

Table 4. Individual Data from Test Session for the Control Group
** Did not reach the learning criterion and was not included in the main analyses*

Animal ID	**#26887**	**#27326**	**#27400**	**#27401**	**#27075***	**#27077***
Successful Trials	75	46	78	63	15	13
Total Possible Trials	75	57	79	69	41	39
Missed Trials	0	11	1	6	26	26
Failed Trials	0	0	0	0	0	0
Stimulated	75	46	78	63	15	13
Proportion of Trials Correct	1.0	0.807	0.987	0.913	0.366	0.333
Successful Trials per Minute	2.538	1.514	2.597	2.068	0.499	0.433
Ball 1 Latency	5.086	12.502	4.594	7.737	24.384	25.675
Ball 2 Latency	2.069	2.875	1.608	3.389	6.601	4.078
Average Trial Length	7.154	15.377	6.201	11.127	30.985	29.753

Table 5. Descriptive Statistics after Inclusion of Non-Learners

In the control group, there were two animals that did not reach the learning criterion of >75% correct trials within seven sessions. Except for the group comparison of how many in each group that reached the learning criterion, these non-learners were excluded from the analyses. Like the rest of the sample, they underwent an additional fully automated test session without manual stimulations given by the experimenter. The table shows descriptive statistics of the control group from the test session when including the two non-learners and statistics from only the non-learners.

	Accuracy	**Latency 1st ball (seconds)**	**Latency 2nd ball (seconds)**	**Average Trial Length (seconds)**
Total control group (n=6)	*M* = 73.44 *SD* = 30.61	*M* = 13.33 *SD* = 9.50	*M* = 3.44 *SD* = 1.79	*M* = 16.77 *SD* = 11.03
Non-learners (n=2)	*M* = 34.95 *SD* = 2.33	*M* = 25.03 *SD* = 0.65	*M* = 5.34 *SD* = 1.78	*M* = 30.37 *SD* = 0.87

Mann-Whitney U tests comparing the two groups, including the two non-learners in the control group, on measures from the test session, showed no significant difference on either accuracy (U = 6.0, p = .257), latency from when the first ball lit up until it was pushed (U = 7.0, p = .352), latency from the first to the second ball was pushed (U = 5.0, p = .171), nor average trial length (U = 7.0, p = .352).

Histology of the Experiment Group

The immunohistochemical labeling of #27037 was performed by Merethe Andresen, while the remaining sectioning, Nissl staining and immunohistochemical labeling was performed by myself.

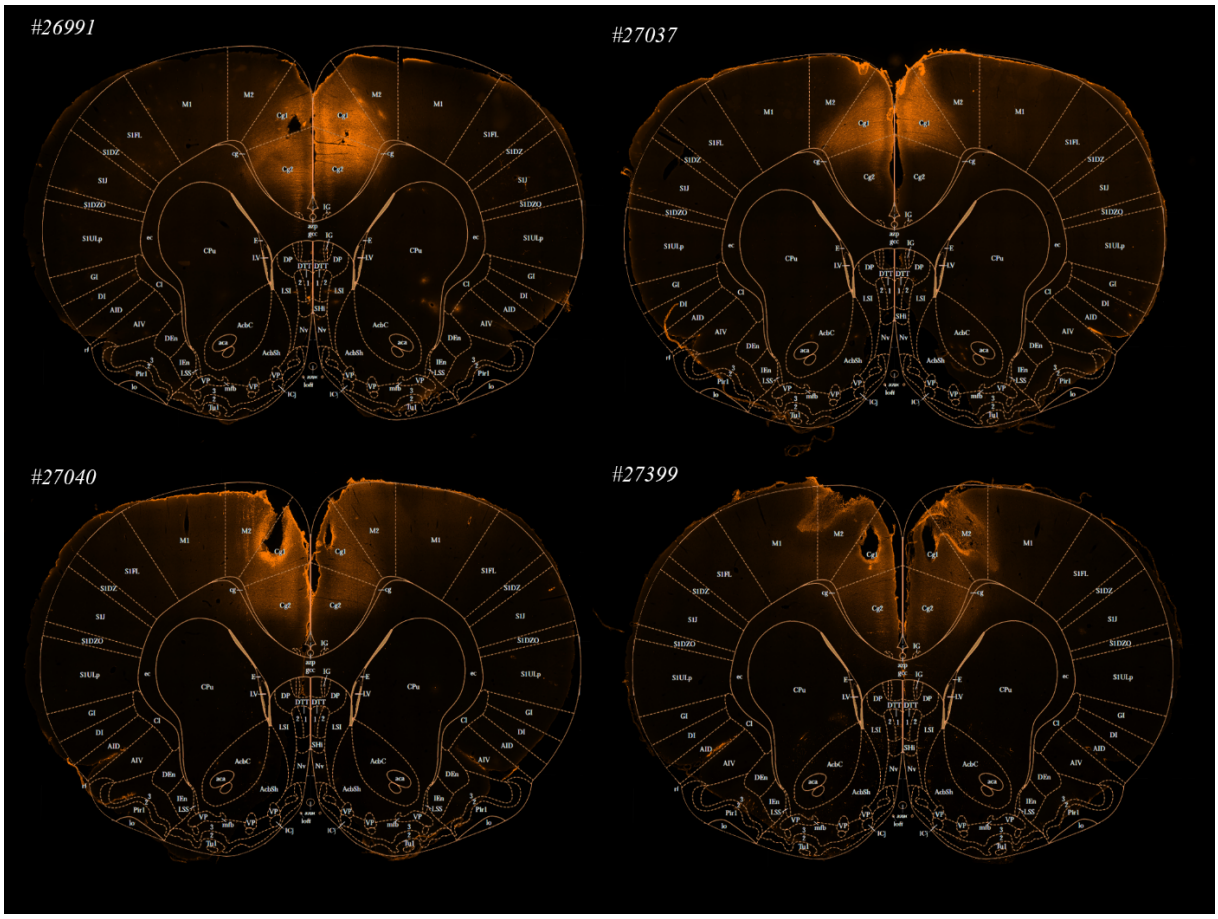
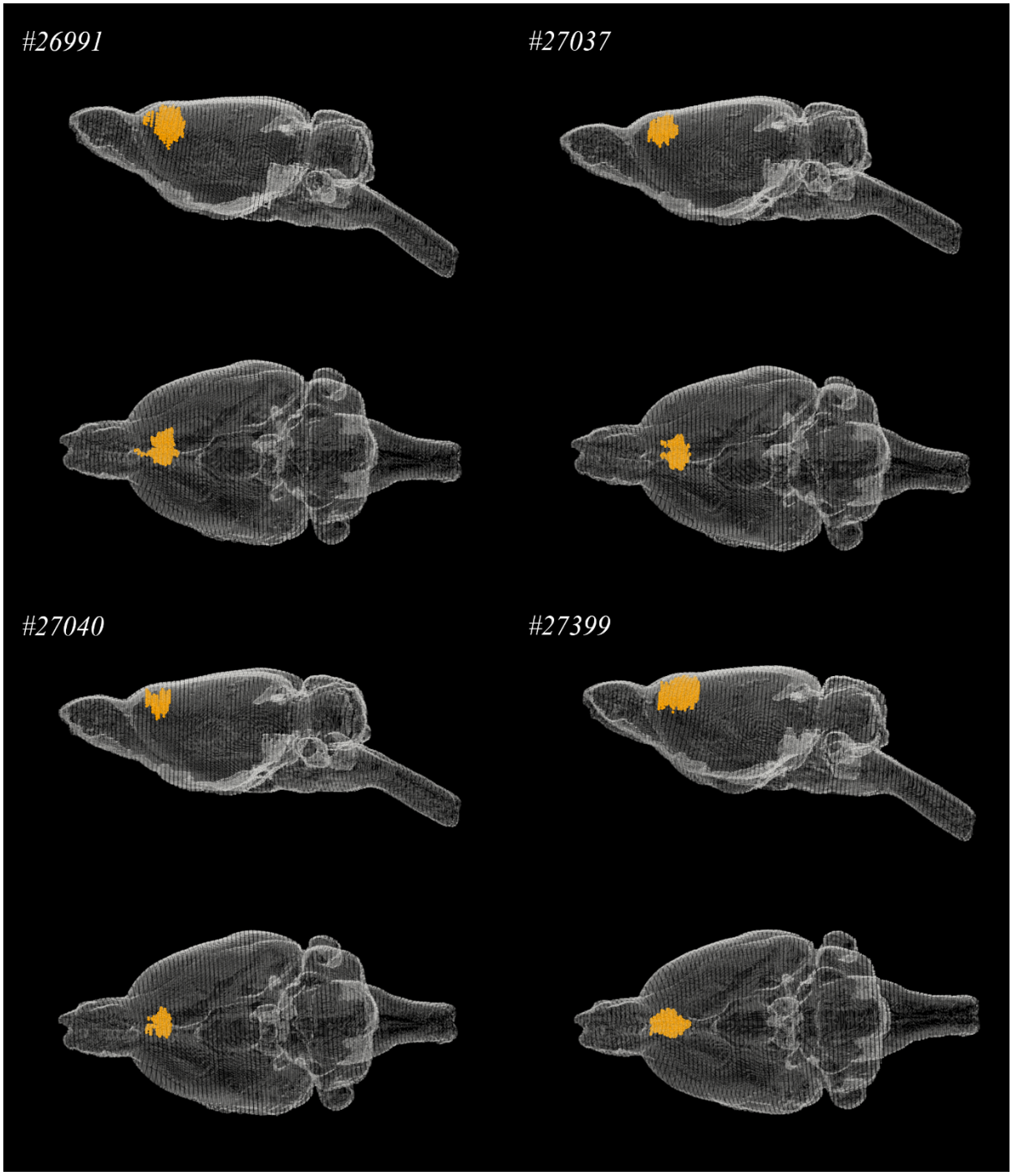


Figure 1. Immunohistochemistry. The figure includes histology for all animals in the experiment group. The mCherry in the viral solution were immunohistochemically labelled and scanned with 546 nm wavelength light. The overlay is from the Rat Brain by Paxinos & Watson (2007) at AP: +2.28.



Figur 2. The reconstruction of the virus expression in TRACER. The software TRACER was developed by the Whitlock group (Paglia et al., in progress) to track the location of probes. It was modified for the use of visualization of virus expression for this project. The figures are captures of the 3D illustration of all experimental animals.

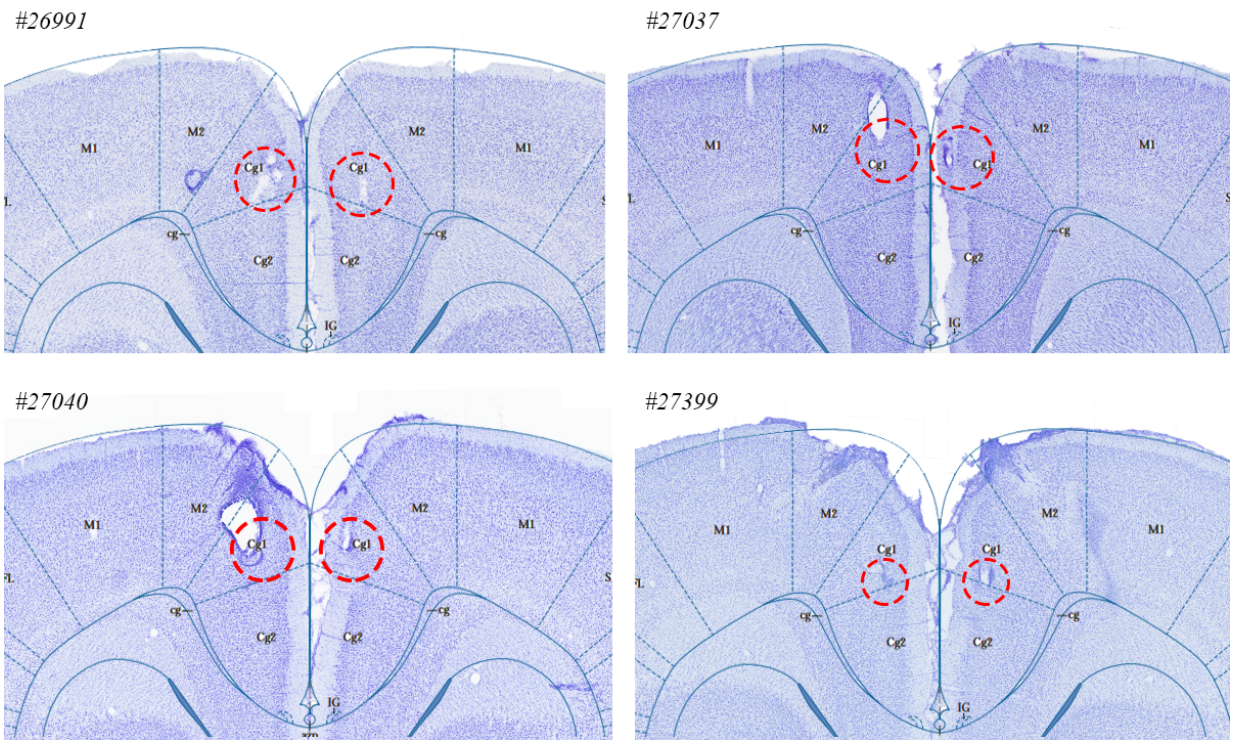
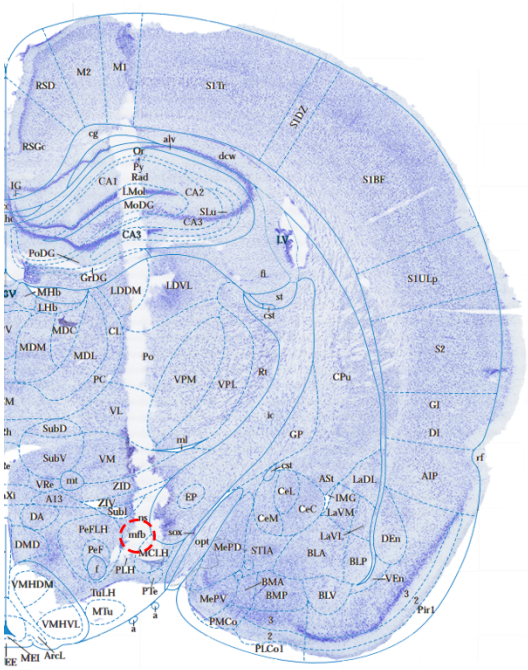
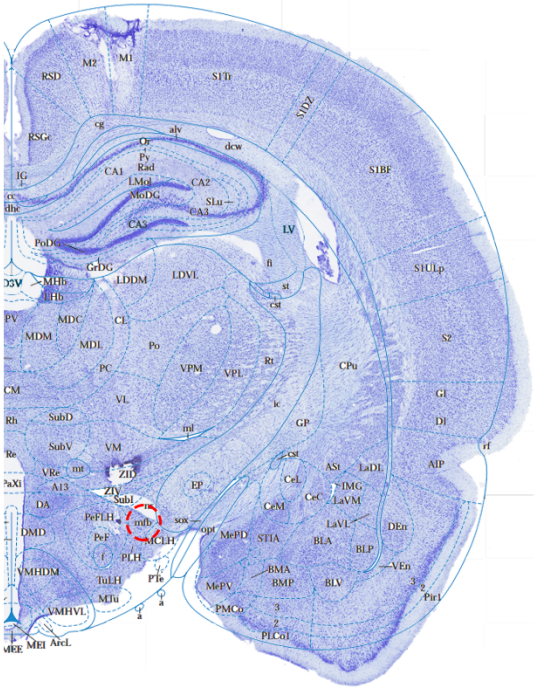


Figure 3. Nissl Staining with Fiber-Optic Cannulas Trace. The figure includes sections from all animals in the experiment group, showing the traces of the fiber-optic cannulas. The circles indicate the end point of the cannulas visible in the section. The overlay is from the Rat Brain by Paxinos & Watson (2007) at AP: +2.28.

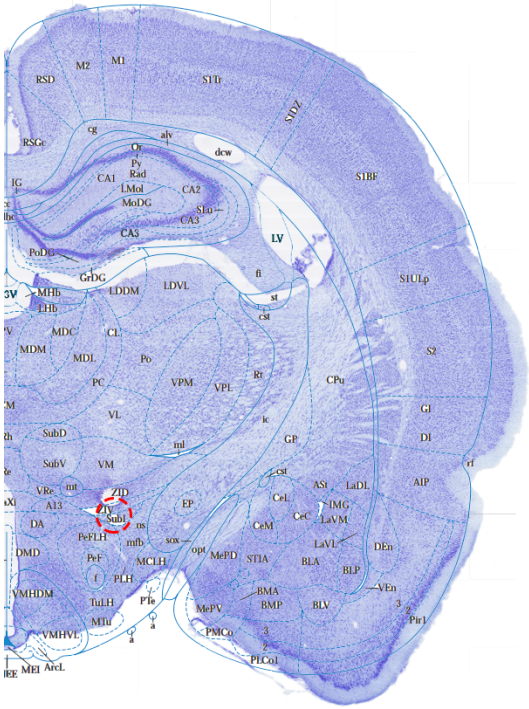
#26991 AP: -2.76



#27037 AP: -2.76



#27040 AP: -2.76



#27399 AP: -1.56

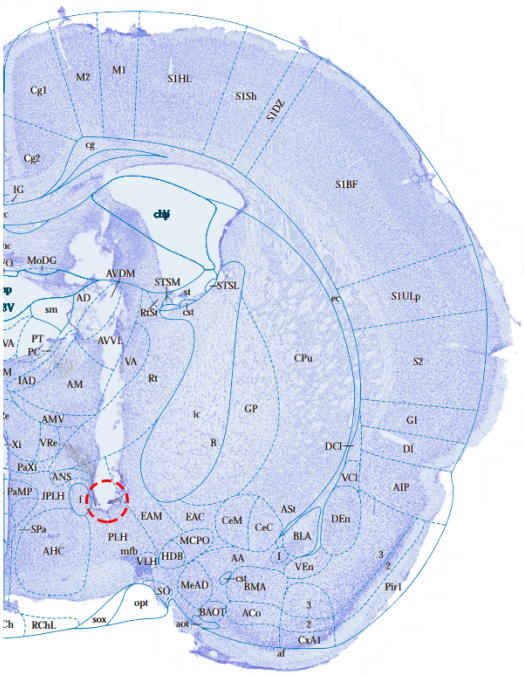
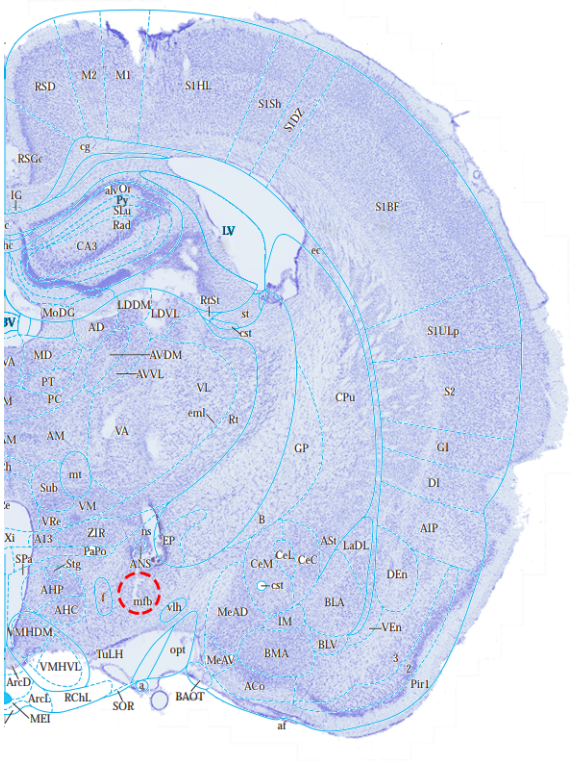


Figure 4. Nissl Staining with Bipolar Stimulating Electrode Trace. The figure includes sections from all animals in the experiment group, showing the traces of the bipolar stimulating electrode targeting the medial forebrain bundle. The circles indicate the end point of the electrode visible in the section. Notice that it does not always reach the medial forebrain bundle (MFB) according to the atlas, but the placement was confirmed as the animal responded to stimulation with displaying reward. The electrode of #27399 use an overlay of AP: -1.56, while the remaining use -2.76, as the trace in #27399 was located more anterior than expected.

Histology of the Control Group

The sectioning and Nissl staining of #27075 and #27077 was performed by Devika Kurup, the sectioning and Nissl staining of #27400 and #27401 was performed by Merethe Andresen, while the remaining sectioning and Nissl staining was performed by myself.

#27400 AP: -2.04



#27401 AP: -2.04

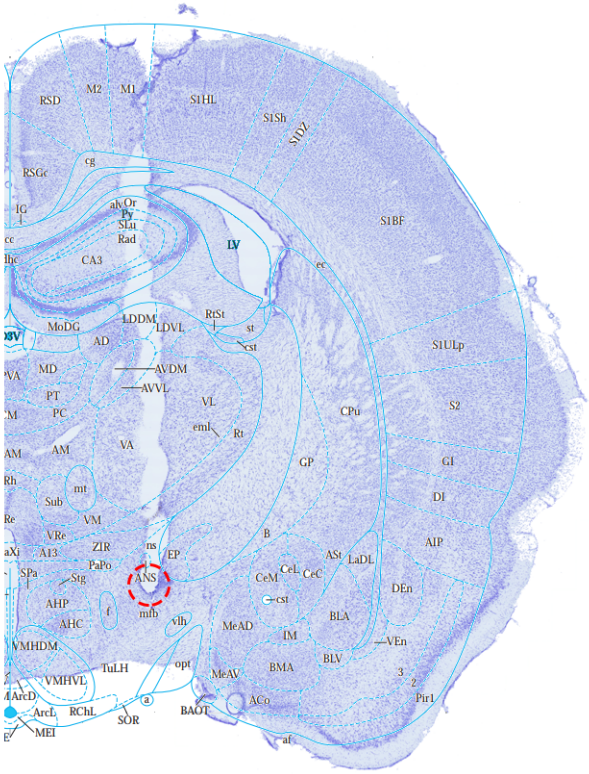


Figure 5. Nissl Staining with Bipolar Stimulating Electrode Trace. The figure includes sections from animals in the control group, showing the traces of the bipolar stimulating electrode targeting the medial forebrain bundle. The circles indicate the end point of the electrode visible in the section. Notice that it does not always reach the medial forebrain bundle (MFB) according to the atlas, but the placement was confirmed as the animal responded to stimulation with displaying reward. The figures use an overlay of AP: -2.04, as the trace was located more anterior than expected.

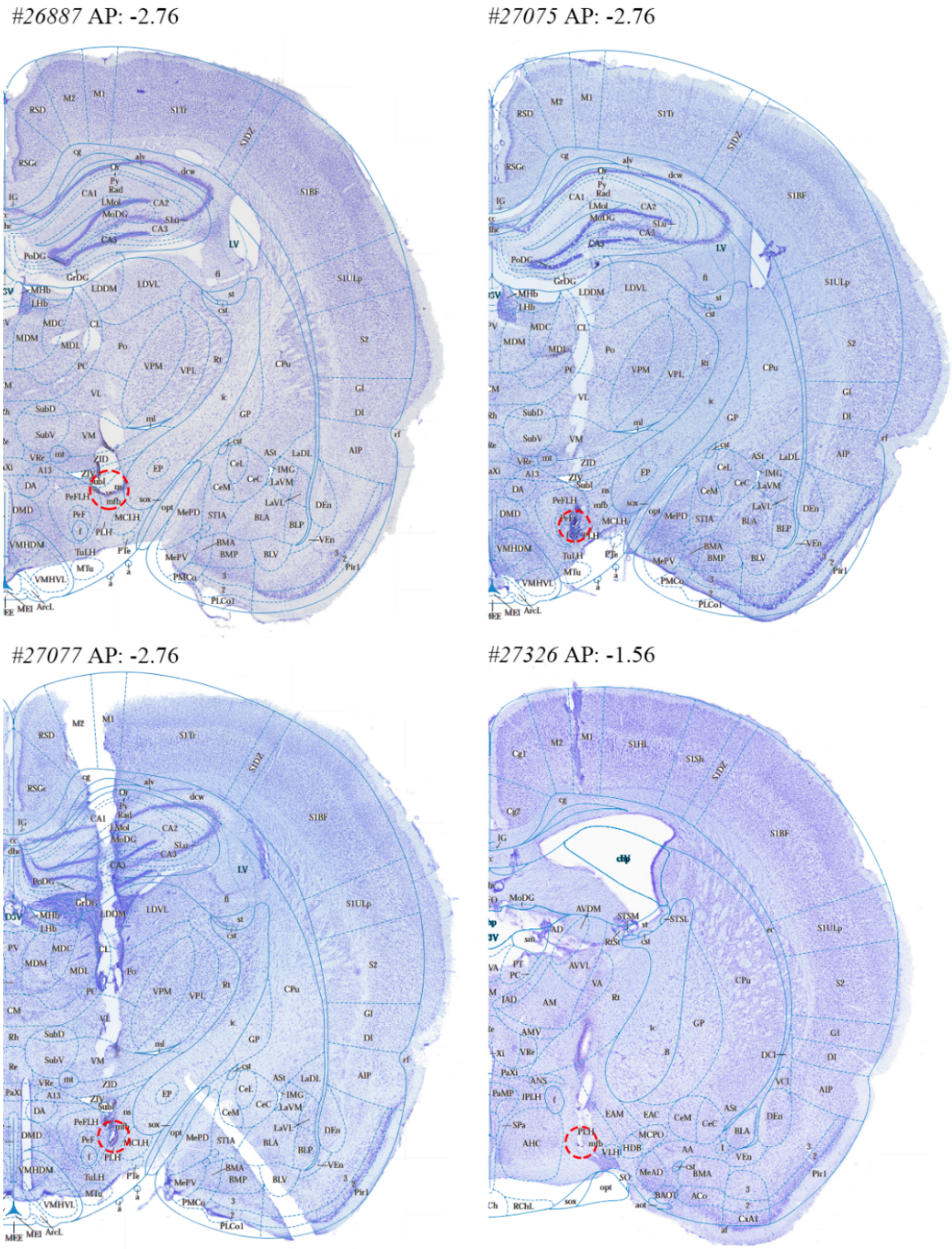


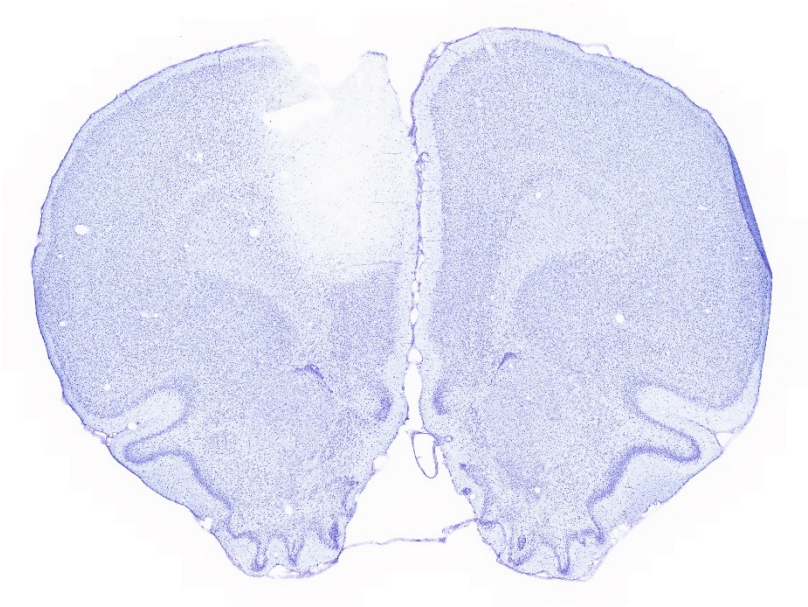
Figure 6. Nissl Staining with Bipolar Stimulating Electrode Trace. The figure includes sections from animals in the control group, showing the traces of the bipolar stimulating electrode targeting the medial forebrain bundle. The circles indicate the end point of the electrode visible in the section. Notice that it does not always reach the medial forebrain bundle (MFB) according to the atlas, but the placement was confirmed as the animal responded to stimulation with displaying reward. The electrode of #27326 use an overlay of AP: -1.56, while the remaining use -2.76, as the trace in #27326 was located more anterior than expected.

Development of the Optogenetic Protocol

Before this project began, the efficacy of functional inhibition of neurons using Chr2 targeted at GABAergic interneurons was tested by Ida V. Rautio, confirming that the viral vector was efficient. For the additional development of the protocol for optogenetic inhibition, three animals were injected with the viral vector and implanted with a fiber-optic cannula and a Neuropixels probe to record the neural activity while using light stimulation. One animal (#26805) had a seizure the day after surgery and was therefore euthanized before any recordings or light stimulations could take place.

Merethe Andresen did the sectioning, Nissl staining and immunohistochemical labeling of #26804 and #26735. The remaining histology was performed by the author.

Rat #26804 was exposed to 30 minutes of continuous light stimulation at ~30 mW in one hemisphere, which led to profound cell death.

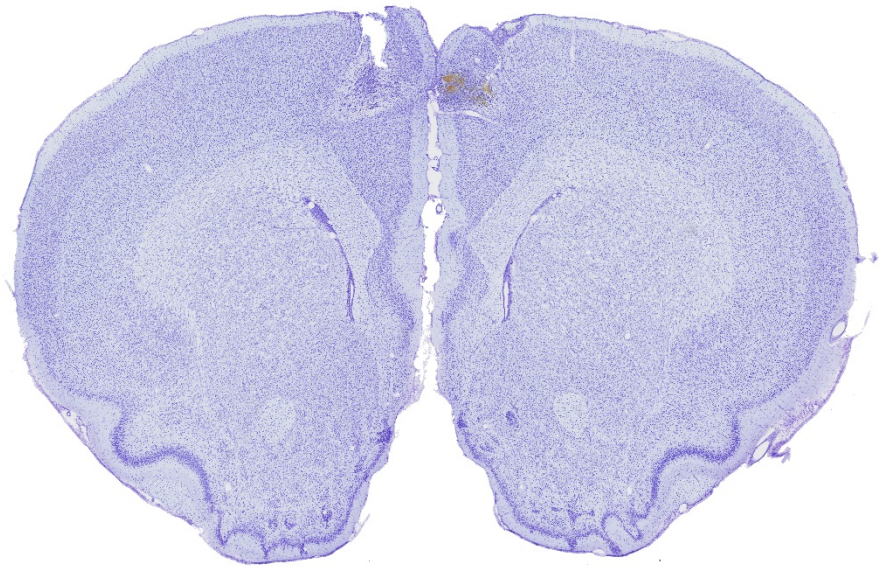


Figur 7. Nissl Staining of #26804. The protocol tested in rat #26804, Longtail, was 30 minutes of continuous light stimulation at ~30 mW. This led to profound tissue damage.

The animal #26735, James, was meant as a pilot animal for the experiment. In addition to a viral injection, the animal was implanted with bilateral fiberoptic cannulas targeting cingulate cortex 1 & 2 and a bipolar stimulating electrode targeting the medial forebrain bundle (MFB). The cannulas used had a distance of 1.4 mm, meaning it was

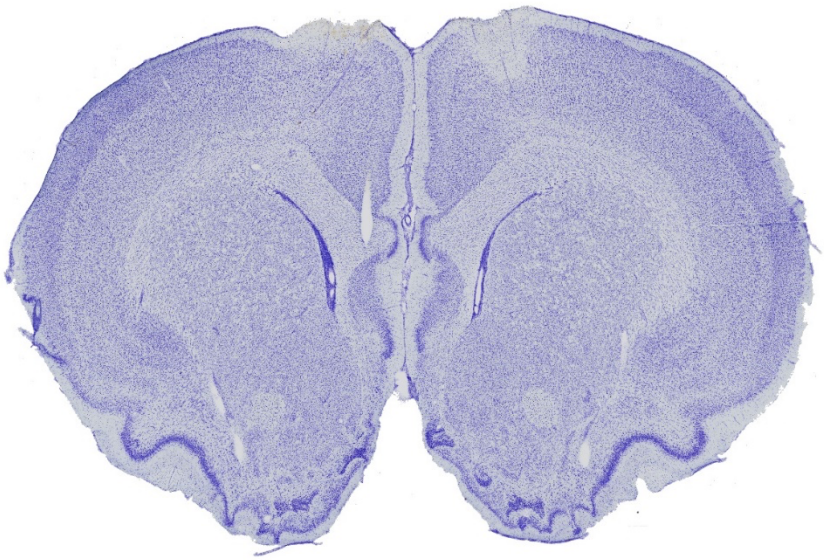
implanted at ML: ± 0.7. The cannulas in the animals used in the experiment had a distance of 1.0 mm.

The histology of rat #26804 was not ready by the time we initiated the pilot sessions with #26735. The rat was therefore exposed to continuous light stimulation at ~30 mW for one 30-minute on five consecutive days. While the histology of this animal also showed some tissue damage, it did not show the same pattern nor was it nearly as encompassing as in #26804.



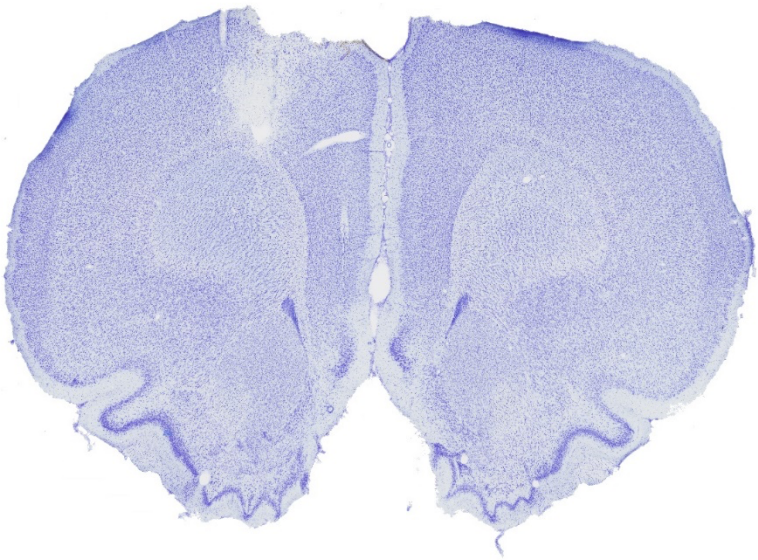
Figur 8. Nissl Staining of #26735. The protocol tested in rat #26735 was five consecutive days of 30 minutes continuous light stimulation at ~30 mW. This led to tissue damage, although not as encompassing as in #26804.

Interestingly, while the rat #26805 was never exposed to light stimulation due to being euthanized because of a seizure, the histology showed some tissue damage, with a similar pattern as but not as profound as #26804. We were unable to explain this, although it might be due to the seizure.



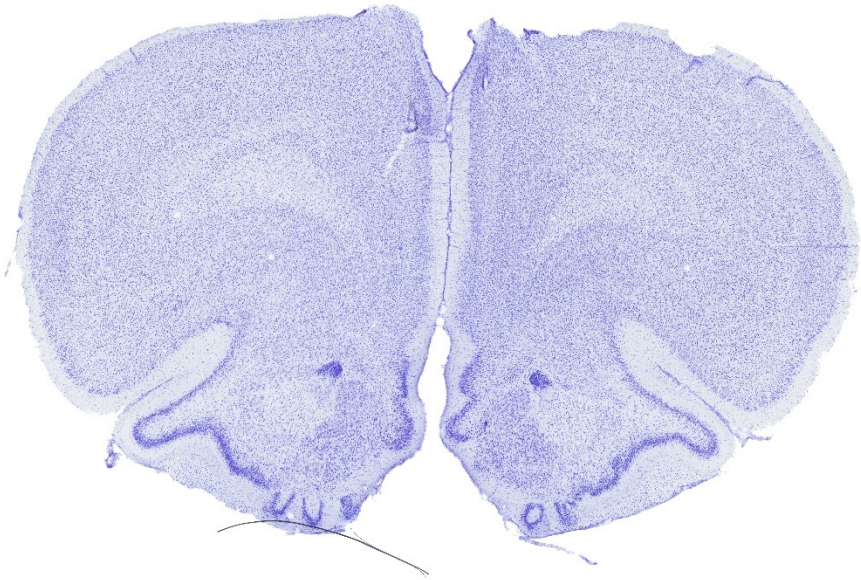
Figur 9. Nissl Staining of #26805. The rat #26805 had a seizure before it was ever exposed to light stimulation and was therefore euthanized. He did, however, show some tissue damage in the same pattern as his littermate #26804.

The rat #26992 was used to test protocols with different pulsating frequencies. Three 30-minute sessions with 60, 40 and 20 Hz at ~30 mW were conducted the same day, in order to keep the cell count for the Neuropixels probe as high as possible. Based on the processing of the electrophysiological recordings, performed by Ida V. Rautio, it was apparent that the cells that were inhibited during the light stimulation returned to their normal firing rate when the laser was turned off. Ultimately, 60 Hz was chosen as it resulted in efficient inhibition of the neurons, with the least signs of entrainment effects. The histology of the animal did however show some tissue damage. This was most likely due to being exposed to the light stimulation for 30 minutes three times in one day, as none of the histology of the animals that was later exposed to 30-minute sessions with 60 Hz pulsating light at ~30 mW on consecutive days showed this tissue damage.

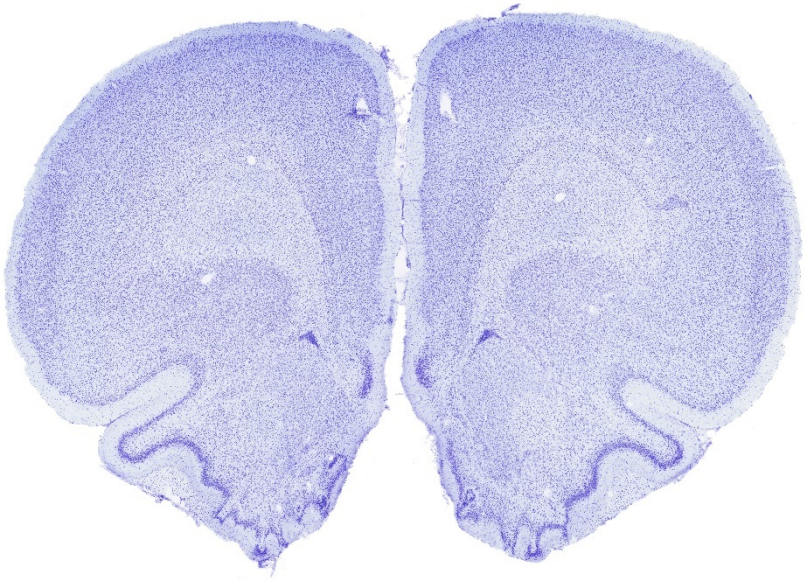


Figur 10. Nissl Staining of #26992. The protocol tested in rat #26992 was three 30-minute sessions with pulsating light at 20, 40 and 60 Hz at ~30 mW. This led to some tissue damage, most likely due to all three protocols being tested the same day.

When we began the actual experiments, two animals, #26993 and #27038, had misplaced electrodes and could therefore not be used in the experiments due to not displaying rewards by stimulations of the MFB. We chose to test the chosen protocol of ~30 mW with 60 Hz pulsation on them to see if any tissue damage was present after multiple sessions on consecutive days. We varied the number of sessions to see when the damage appeared, if it appeared. Rat #26993 underwent two sessions and #27037 underwent seven. Neither of them showed any tissue damage apart from the physical trauma of the fiber-optic cannulas, nor did any of the animals used in the experiment, confirming that 30 minutes with pulsating light at 60 Hz at ~30 mW did not lead to tissue damage when conducted on consecutive days.



Figur 11. Nissl Staining of #26993. The rat #26993 was not used in the experiment due to not displaying reward as a result of stimulations to the MFB. He instead was exposed to 30 minutes of light stimulation at 60 Hz at ~30 mW for two consecutive days. There were no signs of tissue damage apart from the physical trauma of the fiber-optic cannulas.



Figur 12. Nissl Staining of #27038. The rat #27038 was not used in the experiment due to not displaying reward as a result of stimulations to the MFB. He instead was exposed to 30 minutes of light stimulation at 60 Hz at ~30 mW for seven days, with one day break after the fourth day. There were no signs of tissue damage apart from the physical trauma of the fiber-optic cannulas.

NTNU

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Science and Technology