Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Medicine and Health Sciences
Kavli Institute for Systems Neuroscience

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Reward-based observational learning: Importance of social information and Anterior Cingulate Cortex for attention

Master's thesis in Neuroscience
Supervisor: Jonathan Whitlock
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Abstract

Observational learning is a natural behavior that is conserved from rodents to primates, yet the precise underlying neuronal substrate remains unclear. Neurodevelopmental disorders like autism have been associated with deficits in observational learning. Therefore, there is a clear translational interest to characterize these pathological changes. The purpose of this project is to shed light on the role of social information processing and the anterior cingulate cortex in attention and how it could potentially affect observational learning. To investigate this, a non-intuitive behavior paradigm was used. The first experiment was designed to test if rats (observers) could learn to choose a rewarded light ball by observing a performer rat executing the same task. It was established that, in contrast to naive animals, observer rats were significantly faster at learning the task. To test the importance of social information for correct acquisition of the task, we introduced a naive rat in place of a trained performer, effectively removing task-relevant information but still providing a social cue. The results revealed that the observers could not learn the task for this condition, suggesting a critical role of task-specific performance on the part of performer animals during observational learning. Since attention could plausibly contribute to the efficacy of observational learning, we checked for differences in the attentiveness of the observers across conditions. To categorize this, we developed an unbiased analytical tool that incorporated a custom-made behavioral grouping and rodent head-tracking software. The preliminary data indicate that the observers were equally attentive in all experiments, yet they only learned the task in the presence of task-relevant information received from the performer. Finally, to check the role of the anterior cingulate cortex in attention and observational learning, optogenetics was employed to suppress the activity of that region in observers. Preliminary results showed that anterior cingulate cortex inhibition disrupts observational learning since observers could not learn the task through observation. In addition, we report that inhibition of the anterior cingulate cortex does not affect the social attention of rats. Based on these observations, we speculate that the anterior cingulate cortex could be important for processing some form of social information that promotes observational learning but does not disrupt the attentiveness of the animal towards social cues.

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Contents

Abbreviations

ACC: Anterior cingulate cortex

MFB: Medial forebrain bundle

mPFC: Medial prefrontal cortex

M2: Secondary motor cortex

Cg1: Cingulate cortex 1

Cg2: Cingulate cortex 2

ChR2: Channelrhodopsin2

AAV: Adeno-associated viruses

IC: Intracranial

AP: Anterior posterior

ML: Mediolateral

DV: Dorsoventral

ICSS: Intracranial self-stimulation

N-zone: Near-zone

D-zone: Distal-zone

SB1: Stimulus ball 1

PL: Prelimbic cortex

SB2: Stimulus ball 1

Obs: Observation group

Con: Control group

OB: Observer

PF: Performer

TSET: Time spent executing task

PST: Proportion of successful trial

RAQ: Rodent attention quantifier

AF: All frame

LO: Lights ON

RBV: Right binocular vision

LBV: Left binocular vision

NaN: Not a number

A: Attention

PA: Partially attention

PI: Partially inattention

I: Inattention

SD: Standard deviation

Section 1

Introduction

1.1 Learning - a key to survival

Gaining knowledge through experience, observation, and teaching is a fundamental behavior for all social beings. The sole purpose of something as fundamental as cell division is to pass down information across generations. Learning is a process of acquiring new knowledge or behavior(s) after gathering information through interacting with one's environment. This requires a memory trace of an event that can be recalled by the animal at relevant situations (Laland *et al.,* 2020). There are many forms of learning that can be grouped into habituation, imprinting, associative learning, social learning, exploratory learning, and insight learning as explained in more detail with Figure 1.1. Focusing on the current study, this paper will be addressing social and observational learning (Hilliard 2003).

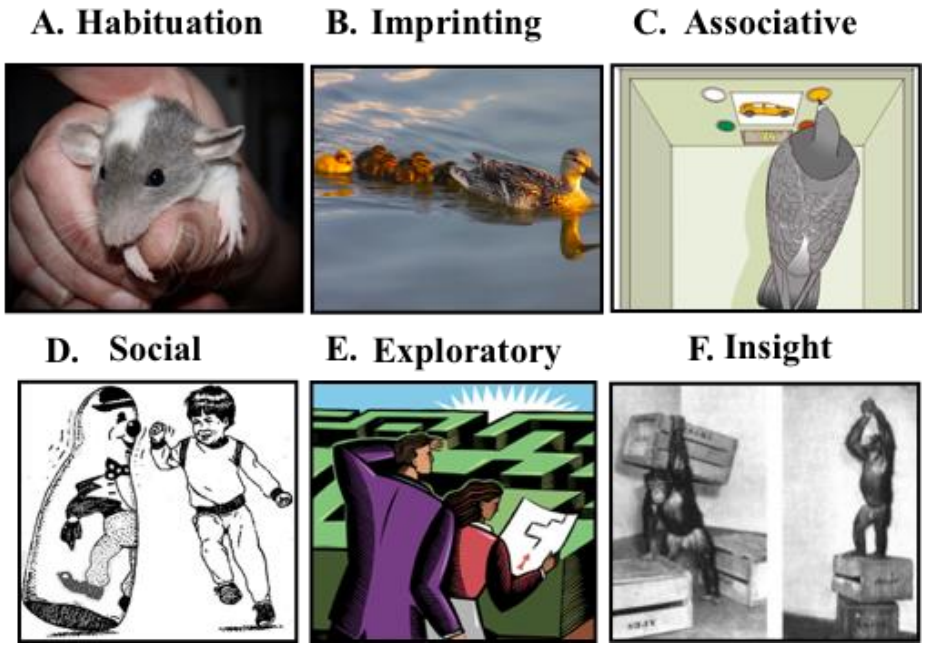

Figure 1.1: **A.** Habituation: a decrease in natural repose after repeated exposure of the stimulus, e.g. rats habituating to human touch; **B.** Imprinting: irreversible learning that is specific to a time period of the animals life, e.g. ducklings that follow mother; **C**. Associative learning: learning to associate one event or stimulus to the result, e.g. any classical and operant conditioning task; **D.** Social learning: learning to associate one event or stimulus to the result, e.g. Any classical and operant conditioning task; **E.** Exploratory learning: learning to make spatial relations to objects in animals' surroundings, e.g., find ways through maze; **F.** Insight learning: learning to combine precious knowledge to solve current problems, e.g., monkeys figuring out a way to increase height in order to reach a food reward (Based on explanation by Hilliard, 2003; Image acquired from pixabay.com and Hilliard, 2003)

1.2 Observational learning

For a social being, learning facilitates survival, adaptability, behavioral development, and understanding of societal norms (Darwin 1896, Hilliard 2003, Laland *et al.,* 2020).

Observational learning is an integral part of social learning that promotes learning through the observation of the actions of others. The link between observational learning and survival has been discussed as early as the 1800s (Wallace 1870, Darwin 1896). One of the earliest notes on observational learning was made in a letter sent by Hon. Daines Barrington about his observation on singing patterns of birds (Barrington 1773). As an experiment, he shifted a subject bird from its home nest immediately after birth, to an environment with other species of birds. He noticed that the subject bird adapted to the singing of its current environment and did not stick to the instinctive notes of its species. However, if the bird was shifted a few weeks after its birth, it had already learned the notes of its species and took a longer time to adapt to the new environment. This experiment was quoted as a piece of evidence for birds imitating a skill rather than acquiring it by birth (Wallace 1870).

Darwin, who observed the adaptiveness of apes against a few 'warning signs in nature' (like colors of poisonous frogs and berries, or shape of snakes) through observing their parents and companions, had suggested that 'imitation' and 'the need to learn' to be an integral part of this form of learning (Darwin 1896). Another early account of animals learning through observation was made by Syunzo Kawamura, where he expanded on the knowledge of the behavior of macaques to learn basic survival skills from parents and siblings (Kawamura 1959). His observations of the famous female Japanese macaque Imo showed how a new behavior was formed and learned across the community. Imo started to wash sweet potatoes in sea water to clean it and improve its taste. This peculiar behavior was soon noted by her siblings, other juvenile macaques, parents and eventually spread to the whole society. Once Imo and her playmates reproduced, this skill was taught to the new generation and thus the behavior spread.

However, it was not until the proposal of **social learning theory**, an idea presented by Albert Bandura and colleagues in 1961, that observational learning became well defined. The first explanation of what observational learning means and how it might influence human behavior was demonstrated by the 'bobo doll experiment' done by Albert Bandura and colleagues in 1961 (Bandura *et al.,* 1961). They demonstrated that preschool children learned by observing adult behavior. This was famously shown by demonstrators assaulting an inflatable doll in front of the children, who after observing this act started exhibiting aggressive behavior towards the doll when they later got the opportunity to interact with it. This was in stark contrast to the control group who had not witnessed any aggression towards the doll and did not hurt the doll when given the same opportunity to do so. The experiment revealed how drastically behavior can be shaped through observation and social transmission. Albert Bandura's theory brings into consideration how social cognitive factors (like observation, attention, or memory) interact with the environment to impact learning of specific behaviors (Bandura 1977). However, this is not to be confused with observational learning. Observation of the environment is a major part of social learning theory and is a critical aspect for explaining how the animal learns, but social learning also involves other forms of learning like direct learning, where an animal learns through trial and error without social assistance.

Why is observational learning important? In 1979, Varni and team conducted an experiment similar to Bandura's work to study the effect of autism on observational learning. They reported that, when compared to the control group, the autistic children could not acquire task relevant information after multiple sessions of demonstrations, irrespective of their age (Varni *et al.,* 1979). Observational learning is critical for development, social understanding, and learning about everyday skills (Meltzoff & Marshall 2018). Additionally, it was recorded that infants learn through observation at a significantly higher rate at the age of 10 to 12 months (Esseily *et al.,* 2010). This study suggests a

critical period during development when children tend to observe their elders and learn basic life skills. Such studies illustrate that social skills like observational learning are critical for survival and development. Additionally, it has been recorded that defects in these skills can be closely linked to certain neurodevelopmental disorders (like Autism Spectrum Disorder; Varni *et al.,* 1979, Meltzoff & Marshall 2018). Exploring the behavioral and neural basis of skills like observational learning could considerably improve behavioral phenotyping of certain developmental disorders.

Another reason why studies on observational learning are important is due to the fact that this is a conserved behavior across different species. There are many examples of observational learning documented in animals, such as puppies nurtured by cats showing the characteristic feline behavior of licking their paws (Darwin 1896), dogs of the Falkland Islands learning to hunt cattle from one another (Romanes 1884), and hatchlings learning to drink and eat by observing their siblings (Romanes 1884). Studies done on other species like non-human-primates (Tomasello *et al.,* 1987, Cisek & Kalaska 2004, Isbaine *et al.,* 2015), birds (Campbell *et al.,* 1999), fish (Laland & Williams, 1997, Midford *et al.,* 2000), and rodents (Corson 1967, Yamada & Sakurai 2018) suggest that these species can demonstrate various forms of observational learning in controlled laboratory conditions. Moreover, Laland and Williams (1997) reported that guppies, *Poecilia reticulata,* learn to swim through a hole to get a food reward by watching another fish do the same (Laland & Williams, 1997). Research group at University of Wisconsin, showed that juvenile Florida scrub-jays make center patched nests more efficiently when the task was demonstrated by other families of jays at a certain proximity (Midford *et al.,* 2000). Such experiments clearly demonstrate that observational learning is an ability that is shared among many species in the animal kingdom.

1.2.1 Imitation and learning

Observing can lead to learning or *imitation*, a behavior where the animal demonstrates repetition of an observed action (i.e., duplicating the behavior). Examples mentioned previously about birds imitating different species of birds (Wallace 1870) and macaques learning from each other how to clean potatoes in sea water (Kawamura 1959) demonstrates that imitation is a specific behavior that follows observation. However, imitation does not necessarily reflect learning, in that it does not necessarily lead to a permanent change in the specific behavior. This was exemplified in a study by Riopelle (1960). He showed that primates could perform object discrimination better when the performer had errors in their performance as compared to performing perfectly. This suggests that the animals were not just imitating the performer but had learned from the errors of the performer, since if they were only imitating the performer, they would also replicate the observed errors. The change in their behavior was arguably acquired through observational learning and not through imitation. Another example was a study done on chimpanzees that showed how they learned to use a T-bar to get food from a box by observing a demonstrator perform the same action (Tomasello *et al.,* 1987). The control group who did not see any demonstrations of how to use the T-bar was not able to use the tool effectively. It was highlighted that the chimpanzees that learned the task had not imitated the exact movements of the demonstrators, but rather used the observed movements as guides on how to open the box. That is, the observers learned to use the T-bar to retrieve the reward, but the movements used were not identical to the observed performers. From the examples above it can be argued that imitation of an observed behavior is not the same as actual learning, even if the imitation of behavior might give the illusion of learning having occurred. Thus, it is important to differentiate whether the change in behavior is due to the imitation of observed behavior or whether actual learning has occurred.

1.2.2 Role of social information

Social information can be described as the knowledge gained through social contact or instructions. Since observational learning involves the presence of another animal to learn, one can assume the importance of social information necessary for this kind of learning. As demonstrated by Bandura, behavior (like aggression) can be influenced drastically by social information (Bandura *et al.,* 1961, Bandura 1977, Nicol 1995). Another well executed study to learn about the importance of social information, was the work done by Auersperg and team on Goffin cockatoos (Auersperg *et al.,* 2014). They introduced a tool-operated task to observers using demonstrators, who would use a wooden strip (tool) of specific length to extract food (reward) kept at a certain distance in an enclosed cage. One of the demonstrators was a trained cockatoo, while the other demonstration consisted of tools operated by magnets that could be manipulated to complete the task (called 'ghost' demonstrator). All the observers who were paired with a performer could complete the tasks at a greater competence (measured with duration of trial and speed) when compared to the observers paired with ghost demonstrators. Additionally, they noted that the actions used to complete the task by the observer (i.e., tool manipulation) were not identical to the performers' actions, suggesting imitation as an unlikely reason for the apparent learned behavior. They conclude that the more probable reason that learning was achieved was due to social transmission of the task-relevant information. This result was in line with what was reported by Tomasello and team (1987) for monkeys operating T-bars to complete a task (section 1.2.1).

1.2.3 Attention

Another key aspect of observational learning is attention. Attention can be defined as a process of focusing on selective information while filtering out alternative inputs in an environment with competing information (Amso & Scerif 2015). In his book, Darwin talks about an animals' ability to be attentive or stay completely focused on a task to achieve desired results (Darwin 1896). He specifically talks about the attention large cats (like lions) display before pouncing on their prey, and how this behavior develops over time from a playful habit to a hunting skill through social facilitation. In this case, observation of hunting behaviors in society helps the animal survive during adulthood. In humans, inattentiveness and hyperactivity have been understood to be underlying factors for social and observational learning defects in children with attention-deficit/hyperactivity disorder (Hoza 2007). Posner and Petersen (1990) set forth three concepts for the attention system (mainly consisting of dopaminergic and cholinergic neurons in the human brain); 1. There is an anatomical difference between the attention system and its processing system, 2. The system uses multiple networks in the brain, 3. Each of these networks carries out specific functions. These regions are grouped as alerting systems for preparation and alertness (brainstem arousal system and right hemisphere systems), orienting system for spatial observation (focused on primarily the parietal cortex), and target detection for focusing on single modalities (focuses on midline frontal and anterior cingulate cortex; ACC). Consistent with this explanation, a 2011 study found that neural recordings from rat brain showed increased activity of ACC when the reward volume was increased or decreased during an odor recognition task, where the animals had to choose which direction to go based on the odor cue provided in order to receive the reward (Bryden *et al.,* 2011). They postulated that increased activity in ACC was related to the animal paying attention to the changes in reward to update its understanding of the task or to learn the task. The current study attempts to quantify attention in a more intricate manner and study how it is affected during ACC-inhibition in Long Evan rats during an observational learning task (detailed in method section).

1.2.4 Mirror neuron system

Mirror neurons have been linked to imitation, and the presence of these cells in the premotor cortex is suggestive of a role in cognitive function while also supporting observational learning (Zentall 2012). These neurons were first identified by Di Pellegrino and colleagues, when they noticed that many neurons in the monkey inferior premotor cortex were activated both during performing goal-directed hand movements, and while observing a demonstrator perform similar hand movements (Di Pellegrino *et al.,* 1992). They later called these neurons 'Mirror neurons', which were identified as a subset of neurons of the area F5 in the premotor cortex of monkeys (Rizzolatti *et al.,* 1996). Furthermore, mirror neurons were found for mouth and facial movements in different populations of neurons in the F5 region of the monkey prefrontal cortex (Ferrari *et al.,* 2003). The rat mirror neurons systems have been studied, for example by Carrillo and colleagues (2019) using *fear conditioning*, a form of classical conditioning in which animals associate an aversive stimulus with a cue or location. This study showed that neurons in the ACC region of an observer rat fired similarly when getting shocked directly and while witnessing another animal getting shocked (Carrillo *et al.,* 2019). Their findings indicate that a subgroup of neurons in the ACC region of rats might directly respond to observing painful or distressing cues but not while the animal itself receives pain, suggesting the presence of neurons specific to observed pain. Although most experiments focused on motor-based movements (Di Pellegrino *et al.,* 1992, Rizzolatti *et al.,* 1996), and fear conditioning (Carrillo *et al.,* 2019), the possibility of these cells to 'represent' *internal action* like mental imagery of the movements to assist learning, was first interpreted by Jeannerod (1994). That is, these cells could explain the efforts put in by a human to imagine themselves doing that task in order to learn the task. Zentall (2012), further explains the role of these cells in learning (specifically through imitation) when the observer was motivated for the reward. This was explained with an example of hungry quails learning to collect food reward after observing demonstrator birds perform food-collecting tasks. They also reported that this behavior was not noticed in a non-hungry quail, for whom interaction with the demonstrator was not hunger-motivated, hence social information was lost. The current study addresses the question of whether relevant social information assists observational learning when the reinforcing reward is intangible (here, medial forebrain bundle stimulation).

1.2.5 Anterior cingulate cortex

The frontal region of the cingulate cortex (a substructure of prefrontal cortex) is what is referred to as the anterior cingulate cortex; or ACC. In rodents, regions of medial prefrontal cortex (mPFC) have been argued to show functional and anatomical properties similar to primate ACC (van Heukelum *et al.,* 2020). Therefore, this region of the rodent brain is subdivided into cingulate area 1 and 2 (ACC, Cg 1/2), mid anterior cingulate cortex, prelimbic cortex, and infralimbic cortex (Figure 1.2). Recent work has identified ACC, amongst other regions, to be involved in several kinds of observational learning. There are several lines of evidence connecting ACC to fear based observational learning (explained in 1.2.4) (Jeon *et al.,* 2010, Allsop *et al.,* 2018, Keum *et al.,* 2018). A fear-based experiment where ACC was inactivated using 4% lidocaine showed that the ACC is required to express freezing behavior (a common measure of fear expression in rodents) during a fear conditioning experiment, since the ACC lesioned animals did not exhibit fear during testing days (Jeon *et al.,* 2010). A similar experiment where mice were fear-conditioned to foot shocks cued by observing a demonstrator, resulted in impaired acquisition of fear conditioning (lack of measurable fear response or freezing), suggesting a role of ACC in transmission of social information about aversive cues (Allsop *et al.,* 2018). Additionally, that study further suggested that ACC lesions did not affect fear responses

when the animal had personally experienced foot shocks (direct learning) before the lesioning (Allsop *et al.,* 2018). Another recent study by Keum and colleagues showed that mutations of specific genes in ACC interneurons increased observational fear in mice using a fear-conditioning task (Keum *et al.,* 2018). On day 1, they exposed a mouse with mutated neurexin 3 gene (*Nrxn3*) to a chamber, in view of a foot shocking chamber where a demonstrator was given foot shocks at selective times, for a 4-minute conditioning session. On day 2, the observer was placed in the foot shocking chamber and the response was measured. The results demonstrated that the animals with mutated ACC interneurons showed increased observational fear on testing day as compared to control strains.

Less work has been done to study the role of ACC in learning when the reinforcer has a positive value (Jurado-Parras *et al.,* 2012, Yamada & Sakurai 2021). One such study on non-human primates used a visual cue reward-prediction task, that assigned different rewards to specific cues that would be displayed on a screen (for 500ms) placed in front of a head fixed monkey (Hayden *et al.,* 2009). ACC neurons were recorded while the animals performed the task. It was concluded that ACC predicted reward since the firing patterns observed when receiving reward cues were similar to when the animal experienced receiving these rewards. While investigating neural processing involved in reward and punishment (through experience and social information), Schneider and colleagues showed that ACC was involved in both reward and fear-based conditions in rodents (Schneider *et al.,* 2020). The experiment involved two observer rats performing a classical conditioning (mechanism of associating previously neutral stimulus to reward) task, where auditory and visual cues provided information on whether the session ends with either a reward, punishment, or nothing. ACC neurons were recorded from one of the rats (recorded observer), and the results showed that specific subgroups of ACC were involved in modulating outcome identification and outcome prediction. Additionally, ACC responded equally to information on reward or punishment (using auditory cues), which led them to suggest a role of ACC in attention. An observational learning experiment with mice reported that stimulating the mPFC of the observer exactly when the performer pressed the lever (the task, in this case which leads to food reward), inhibited observational learning (Jurado-Parras *et al.,* 2012). Building on this, more recent work done in rats showed similar results (Yamada & Sakurai 2021). In this particular experiment, an observer was allowed to freely observe a demonstrator who was trained to escape a Barnes maze task (where the animal had to choose the correct well or hole to escape). After two days of observation, where the observer received intracranial stimulation at mPFC exactly when the task was performed, the observer was tested for learning. They reported that stimulation of mPFC during task observation disrupted observational learning of the task, suggesting a critical role of rat mPFC in observational learning. It is also important to note that ACC plays a major role in modulation of other processes like emotions, attention, autonomic response regulation, and cost-benefit analysis (Schweimer & Hauber 2005, van Heukelum *et al.,* 2020), and hence cannot be restricted to just observational or social learning.

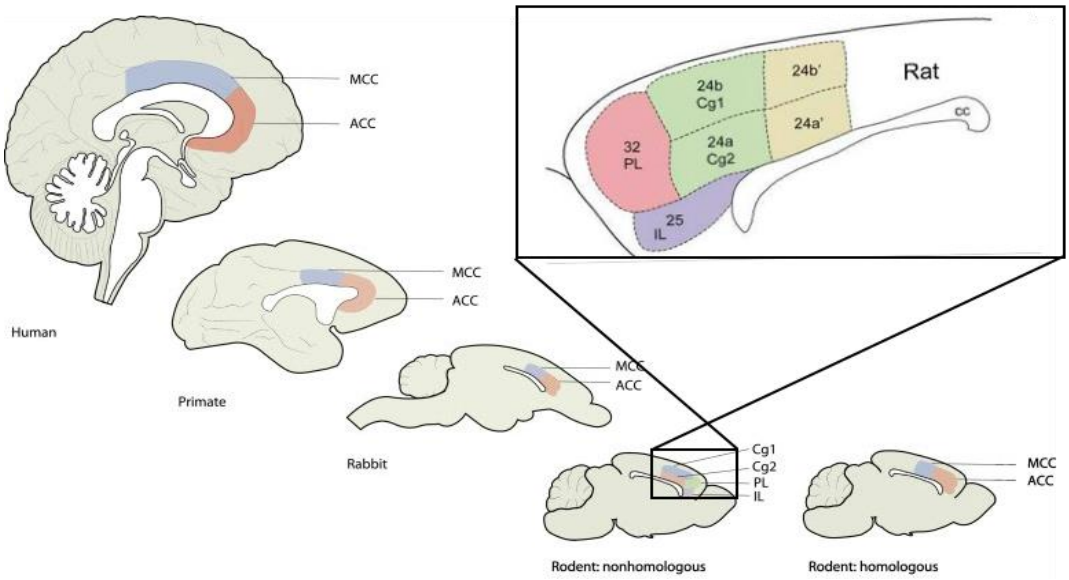

Figure 1.2: Illustration of ACC, that is conserved across species (taken from van Heukelum *et al.,* 2020, image inside taken from Burgos-Robles *et al.,* 2019). ACC: anterior cingulate cortex; MCC: mid anterior cingulate cortex; Cg1: cingulate area 1; Cg2: cingulate area 2; PL: prelimbic cortex; IL: infralimbic cortex.

As mentioned, the ACC is involved in various processes that may be important for observational learning. The current project therefore aims to study the effect of ACC inhibition on attention and learning in a reward-based observational learning paradigm.

1.3 Rodent behavior analysis for observational learning

Bandura explained observational learning as an acquisition of a certain style of thinking and behavior through observation of the examples provided by others (Bandura 2008). He further pointed out that a basic model for observational learning would include: an observable model (digital or live) and intrinsic reinforcement - a method that strengthens behavior through reinforcers and the internal need to learn (Bandura 2008, Nabavi 2012). To study an intrinsic event like observational learning, it is important to define what it means to learn through observation. Banduras' explanation of this process was a rudimentary definition that could be further expounded upon, as done by Zentall (2012). In his review on observational learning, Zentall defines this process as a change in behavior following an observation of a model performing a similar behavior or observing the product of the behavior. This definition captures the range of possible factors that can lead to learning. The motivation to learn through observation may vary greatly depending on multiple factors like; the productiveness of the behavior, certainty of outcome, cost of learning, and even the identity of the model or performer used (Zentall, 2012). Moreover, Zentall emphasizes the effect of social influences in learning, like the relationship between the observer and the model, age, and behavior of the model. Apart from the model, other variables to consider are:

1. External cues: Depending on the experimental design, environmental cues like odor, visual cues, and sound can be controlled through habituation (Laland *et al.,* 2020).
2. Conditioning using reinforcers or punishers: The way an experiment is designed to provide reinforcement makes a difference in the learning of a task. Motivation can be increased using positive reinforcement like food reward (Carlier & Jamon 2006)

to reinforce a behavior. Here, *reinforcers* are used to describe something as being a consequence that makes a behavior more likely to be repeated, and *punishers* as having an effect that makes a behavior less likely to be repeated.

An article on behavioral analysis of rodents recommends specific strategies to use while conditioning rodents to behavioral experiments (Sousa *et al.,* 2006). To conduct behavioral tasks intended to explore learning, experimenters are recommended to (i) allow the animal to explore the arena before providing experimental stimuli to reduce stress and permit them to return to their behavioral baseline (signs of relaxation are often considered to be behavior like grooming, laying down, or bruxing), (ii) reduce external cues to a minimum and keep conditions consistent throughout the entire experiment and across sessions, (iii) use of videos to record the behavior of the animal in the experiment apparatus, and (iv) habituate the animals to sensory cues that are an unavoidable part of the experimental design (e.g. sounds of experimental devices or monitor screen lights).

Analysis of the observed behavior is an integral detail to examine and validate results of behavioral tasks. As mentioned in section 1.2.3, for a behavior like observational learning, quantifying attention is critical. Without unbiased analysis for attentiveness, results suggesting learning through observation (that demands attention of the observer) could be misinterpreted or even errant. Due to the complicated nature of studying attention, rodent experiments often use the orientation of the body relative to the point of interest as a proxy for attention (as seen in Bryden *et al.,* 2011; section 1.2.3).

Tracking a rat's point of visual focus would seem like a logical solution for attention quantification, however, apart from rapid head movements and rotations, freely moving rats can independently rotate both their eyes horizontally up to 40° away from the center of their visual field, and close to 60° vertically (Wallace *et al.,* 2013). Moreover, a rat's overlapping binocular field of vision ranging from the center (or the snout), at horizon level is 40° (Figure 1.3 A), and when the head is tilted up it can be up to 110° (Land 2013). The lower range of rats' monocular vision is 146° while the higher range is 176° (illustrated in Figure 1.3 B) (Hughes 1979). This makes eye tracking effective but complicated, and it does not guarantee that the animal is attending to the point in space where the pupil is pointed (Wallace et al., 2013). A few studies have, however, attempted to analyze attention using rodent body tracking software (Rousseau *et al.,* 2000, Allsop *et al.,* 2018, Lorbach *et al.,* 2018, Carrillo *et al.,* 2019). Rousseau and his colleagues manually analyzed recordings of rat behavior and then trained a neural network to identify seven postures observed in rats (Rousseau *et al.,* 2000). The custom-made software could identify the body posture, head angle, nose direction and tail joints of the animal for each frame and identify the postures with 63.7% accuracy in comparison to a human-annotated (or manual) analysis. Another example of use of a common software, ODLog (a macropad software used for behavioral scoring), comes from the work by Allsop et al. (section 1.2.5), where they used the orientation of the mice to tag startled and freezing responses to quantify attention during fear conditioning tasks (Allsop *et al.,* 2018). Similarly, Carrillo et al. (2019) used open tracking software BORIS to collect data on the head orientation of observer rats with respect to the position of the performer and used this as a measure of attentiveness in their experiments (explained in 1.2.4).

A more recent tool used to analyze behavior of animals was made by Lorbach and colleagues, where they came up with a 'RatSI dataset' software that grouped social behaviors and social interactions (like grooming and attack) between two rats, which helped identify these interactions in session recordings (Figure 1.3 C and D) (Lorbach *et al.,* 2018). The observed behavioral categories were annotated by humans and compared with the behavioral software. In this case, it was reported that the software recognized

certain behaviors with high accuracy (like allogrooming, running, pinning and attacks), while accuracy of other behaviors (like inactivity, freezing, following, and moving away) were not significant. Another impactful development in the field came with the release of DeepLabCut, a deep-learning-model based body-posture estimation tool (Nath *et al.,* 2019). This tool has been widely used to study movement, posture, and animal behavior (Kim *et al.,* 2020, Clemensson *et al.,* 2020). Manual scoring of behavior could be accurate but will be open to criticism due to the probability of human errors. On the other hand, artificial networks that can score behavior are less biased and the quantifications are generally more accepted, though they could lack accuracy in recognizing emotions and specific behavior at the same level as a human might (see for example Rousseau et al. (2000) and Lorbach et al. (2018)). Bridging software- and experimenter-based forms of analysis can help merge these differences and make a more robust quantification of behavior.

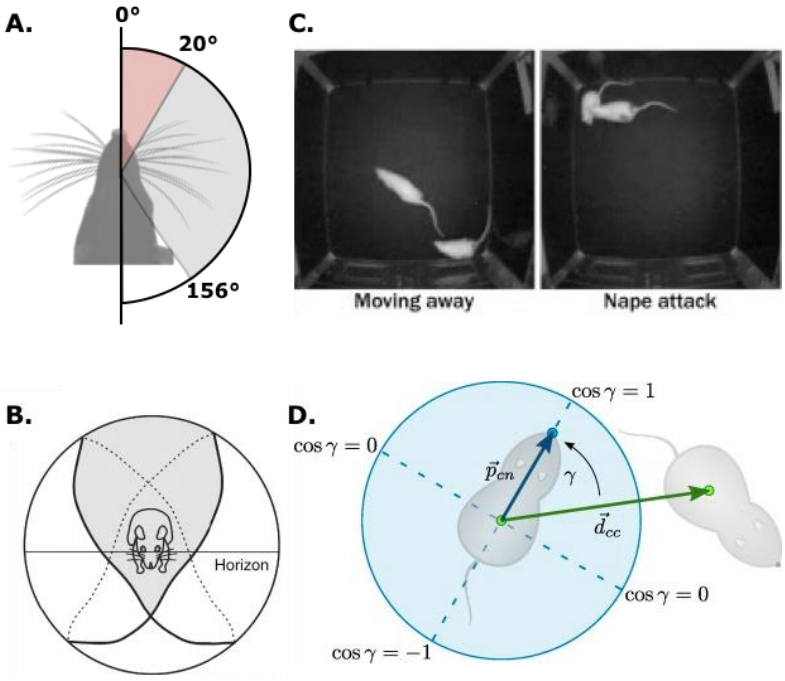

Figure 1.3: A. Representation of the rat binocular field of view (in red), and minimum monocular field of vision (in grey) at horizon level (from Hughes 1979), **B**. Illustration of the wide visual field of rats when moving upwards from the horizon (taken from Land 2013), **C and D**. Example recording of social interactions between rats, and distances and angles calculated using video recordings and RatSI software to identify the form of behavior (taken from Lorbach *et al.,* 2018)

1.4 The current study

It is understood that the ACC is involved in the modulation of various processes that contribute to observational learning (section 1.2.5) and as explained previously, observational learning is a critical behavior for survival that is found across species (section 1.2). Therefore, the current study, which is a part of a larger project, intends to uncover the involvement of ACC in observational learning and visual attention using a reward-based paradigm. The experimental paradigm (designed by Ida V. Rautio as part of a PhD project) consists of a trained performer rat and an observer rat, where the performer executes a sequential ball-tapping task that results for a reward when performed correctly, while the observer is in an adjacent chamber separated by a perforated, clear wall, such that the animal can observe the task at free will. Each of two ping-pong balls with blue LEDs inside are illuminated in a specific order, with the first ball being an unrewarded

trigger which, when tapped in time, causes the second ball to illuminate. If the demonstrator taps the second ball within 30 seconds, both the performer and observer are rewarded. The reward in this paradigm was intracranial stimulation using a bipolar electrode targeting the animals' medial forebrain bundle (MFB), a structure that is part of the brain's reward system. Reward pathways in the rat brain have been studied well by the use of, for example, fluorescence microscopy, to map the neuronal path for reward in the rat brain (Routtenberg 1978). It is observed that the reward pathways extend both ways from the hindbrain, the midbrain and to the frontal cortex, by way of the medial forebrain bundle (illustrated in Figure 1.4). Olds (1958) was one of the first to show that stimulation of brain tissue in general was not enough to induce self-stimulation behavior in rats, but that certain regions of the brain are critical to target for this behavior to manifest – like the medial forebrain bundle (MFB), followed by other researchers (Carlezon & Chartoff 2007).

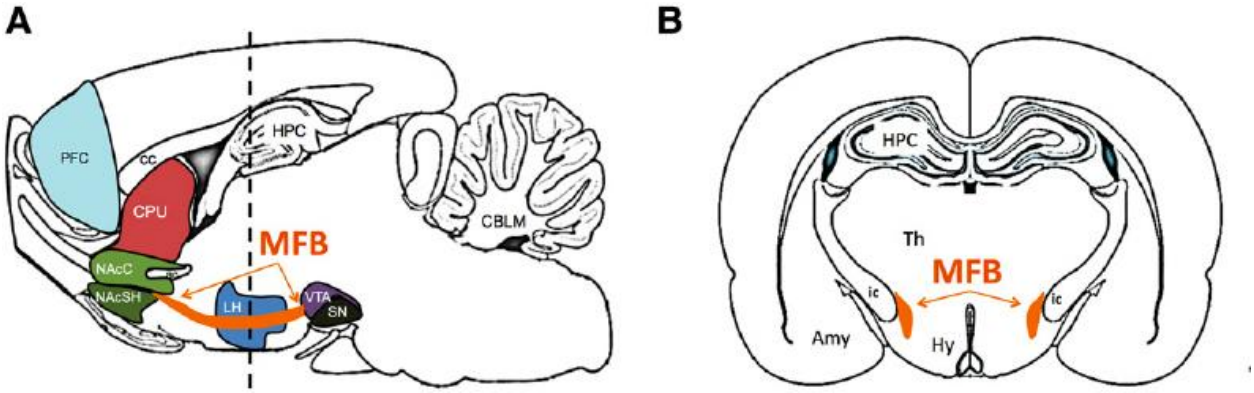

Figure 1.4: Medial forebrain bundle (MFB) in rat brain. **A.** Sagittal section illustrating reward pathway; PFC: prefrontal cortex; NAcSH/C: nucleus accumbens shell/core; LH:lateral hypothalamus; SN: substantia nigra; VTA: ventral tegmental area. **B.** Coronal section of the same, HPC: hippocampus; Hy: hypothalamus; Th: Thalamus; Amy: amygdala; ic: inferior colliculus (image taken from Negus & Miller 2014).

The project in this thesis explores the role of some kinds of social information in the ACC during our observational learning paradigm. Specifically, two experiments were conducted: the observational experiment, where the observer was allowed to observe a trained performer executing the task, and the control experiment, where an observer and an untrained "performer" were allowed to freely move in their respective chambers, while the cues of the task and the reward were delivered automatically, irrespective of the behavior of the naïve "performer". In both cases, the observers were allowed to watch the performers for a 30-minute-long session, and after three such sessions – one session per day on consecutive days - the observer was tested on the task and the results were recorded for analysis. The control experiment was designed to include both social and reward elements of the original task, but to specifically lack the goal-directed behavior of a trained performer to demonstrate the contingencies of the task.

Additionally, we tested the involvement of the ACC in this behavior using optogenetics. To do this we injected a viral vector carrying Channelrhodopsin-2 (a light-gated ion channel) into ACC Cg1/2, which expressed under a pan-interneural enhancer, mDlx, which specifically targets GABAergic interneurons locally in the target area. This resulted in expression of Channelrhodopsin-2 in ACC interneurons. Delivering blue light of wavelength of 473 nm (Lin 2011) by way of implanted optic fibers to the ACC, causes the interneurons to be excited, resulting in inhibition of surrounding principal neurons. Using this approach, we inhibited ACC in the observers during all three observational sessions detailed above before they were tested on whether they learned the observed task or not.

This was done to examine the role of ACC in observational learning, and early preliminary data was used for behavioral analysis here.

The primary focus of the study was to quantify variables that could affect observational learning, specifically, performance of the demonstrator and attention of the observer. The study also sought to automatically quantify attention using a new rodent attention software, and then validate the results by comparing them against a manual behavior analysis method. The goal of these analyses was to compare the effectiveness of different forms of analysis in quantifying the animals' attention and establish which was the more robust method to estimate the attention of the observers.

1.5 Objectives

The current study had three objectives:

1. Control for social information in an already established observational learning paradigm,

2. Determine if the learning deficits seen after optogenetic inactivation of ACC were due to attentional deficits in the observers,

3. Formulate an analytical method to quantify attention of the observer animals, to and apply this method across all experimental groups.

Section 2

Materials and methods

2.1 Animals

The data was collected from 28 adult male Long Evans rats (350 - 450 grams) obtained from Charles River (USA) and bred at Kavli Institute of Systems Neuroscience. Out of the 27, 10 were *performer* animals pre-trained to execute the experimental task, while 17 were naïve animals to be used as *observers* of the experimental task across three sessions (Supplementary table B1 and B2). The housing center had a reversed 12-hour day-night cycle, while food and water were available at home cages ad libitum. The animals were habituated from five weeks of age, and they were housed together with their siblings up until the time of surgery. Post-operatively the rats were housed alone throughout the duration of the experiment while being handled every day to maintain a level of comfortableness with the experimenters and to ensure regular stimulation during single housing. The maintenance and care of these animals were in accordance with the Norwegian Animal Welfare Act and European Convention for the Protection of Vertebrate Animals.

2.2 Observational learning paradigm

The paradigm developed by Ida V. Rautio was designed to study observational learning in rats using a performer animal to complete a pre-trained task and an observer animal to learn that same task through observation. In this paradigm, both animals are implanted with a stimulating electrode targeting medial forebrain bundle (MFB) to deliver a reward when the task was successfully performed. The paradigm features an observer who was allowed to freely watch a performer execute a sequential ball pushing task for a 30-minute-long session once a day for three consecutive days. Both the animals receive intracranial stimulation at the MFB as a reward, each time the performer completed the task (detailed in section 2.4). After the three days of observation, the observers' ability to have learned the task was tested 24 hours after the third session. Based on this paradigm, the three behavioral experiments investigated were the *observational experiment* (trained performer and naïve observer; n=6), *control experiment* (naïve performer and naïve observer; n=6), and *observational experiment with ACC inhibition* (trained performer and naïve observer with inactivated ACC during observation; n=5), further explained in section 2.9. Preliminary data was collected by three researchers: Devika Kurup (two observational experiments and one control experiment), Ella H. Holmberg (three control experiments), and Ida V Rautio (four observational experiments; two control experiments, and five observational experiments with ACC inhibition) (detailed in Appendix C, Supplementary data Table C1).

	Day 1 to 3						Day 4
	Observational (n = 6)		Control (n = 6)		ACC inhibition (n = 5)		All group
	Performer	Observer	Performer	Observer	Performer	Observer	Observer
IC MFB stimulation	Yes	Yes	Yes	Yes	Yes	Yes	Yes
IC self stimulation	Yes	–	No	–	Yes	–	Yes
IC script controlled stimulation	No	No	Yes	Yes	No	No	No
Trained animal	Yes	No	No	No	Yes	No	–
Optogentic inhibition	No	No	No	No	No	Yes	No

Table 2.1: Shows state of animals used for different experiments, namely, observational experiment, control experiment, and observational experiment with ACC inhibition. Every experiment lasted 4 days, with day 1 to 3 being observational sessions and day 4 being the testing day**.** IC MFB stimulation: animals with MFB implants; IC self-stimulation: MFB stimulation received through freely performing the task; IC script-controlled stimulation: MFB stimulation controlled via script and not the animals' actions (independent of the animal); Optogenetic inhibition: animals with fiber optic cannulas where ACC was inhibited only during task performance; IC: intracranial; ACC: anterior cingulate cortex.

2.3 Apparatus

The behavioral experiments were carried out in a transparent box made in-house (L: 100cm W: 40cm H: 70cm). The box was made without a roof to allow for movement of the cords and wires necessary for the set-up and the walls were made of transparent acrylic. The area inside the box was divided into two segments by a perforated barrier made of the same material as the walls, from here on called the *divider*, to allow for multimodal communication, but prohibiting physical interaction between the individual animals in each chamber. The two segments were termed the *performer chamber* (60cm) and *observer chamber* (40cm) as seen in Figure 2.1, A. The performer chamber included two ping-pong balls mounted on top of metal rods with an LED within each of them, where each ball was situated on either side of the performer chamber. These balls acted as *stimulus balls* during the experiment. This was placed on a table in the bird's-eye view of an 8-megapixel Sony IMX219 sensor camera (Raspberry Pi NoIR camera) connected to a Raspberry Pi (Raspberry Pi Org, version 3, UK). The room was kept in a low light setting with infrared light sources to enable video recordings. For the purpose of analysis, the observer chamber was during the analysis process divided into a *near-zone* (spanning 0-20cm from the divider) and a *distal-zone* (spanning 20-40cm from the divider) (Figure 2.1 B and C).

Intracranial stimulation of animals in these chambers was carried out through a programmable pulse stimulator (A.M.P.I. Master 9, USA). The Master 9 was connected to two stimulation boxes (A.M.P.I. ISO-Flex; Microprobes, USA) that had a dial for adjusting the current amplitude while keeping the current constant. Each of the boxes were used to individually control the strength of the stimulation to each of the animals in the box - one in each chamber - independently of each other. The cords (≅6m long) connected to these stimulation boxes were suspended over the box through a commutator and could be connected to the electrode of the freely moving animal when needed. An elastic string was attached to an alligator-clip to allow for adjustment of the length of the cords.

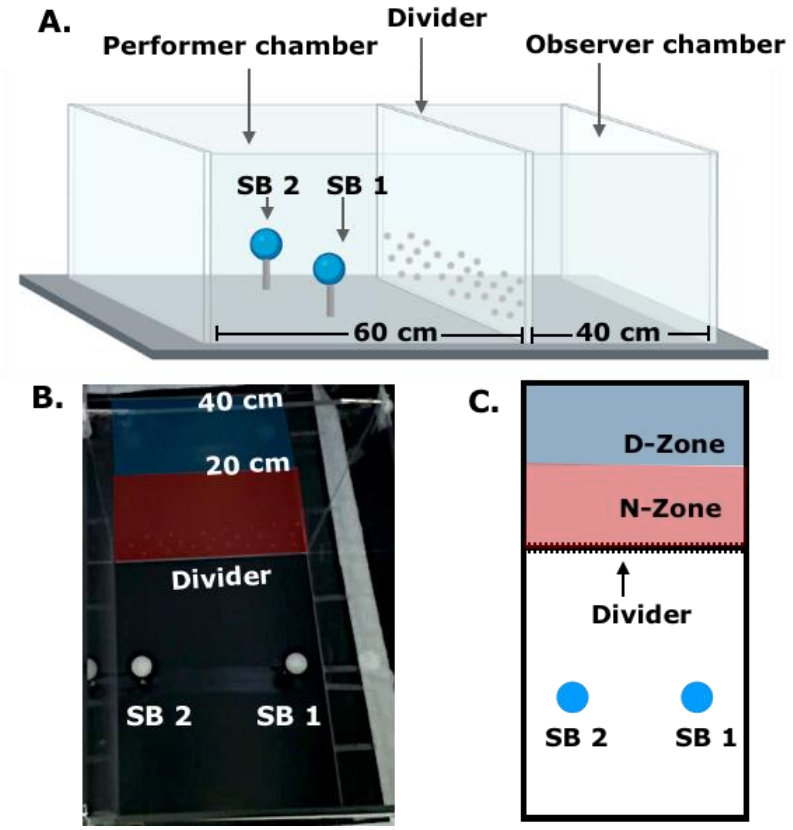

Figure 2.1: The observer chamber was further divided into two segments and only for visualization purposes here depicted with red (*Near-zone*) and blue (*Distant-zone*). During the actual experiments these colors were not present **A.** Schematic view of the experiment box, with SB1 and SB2 representing *stimulus ball* 1 and 2 respectively. **B.** Camera view of the box as seen in video recorded sessions. **C.** Overhead schematic view of the setup.

For optogenetic experiments, two fiber-coupled light sources of 430-490nm wavelength (131mW ND laser and 170mW laser, Sloc lasers, China), were used that provided the blue-light photostimulation (473nm) necessary for the inhibition condition. These were powered by DPSSL drivers (Sloc lasers, China). When the lasers were turned on, each of the light beams passed through individual dimmer wheels and were reflected by mirrors into two individual collimators. The collimators focused each of the light beams into dual-fiber patch cords, while the wheels placed in the laser beam path were used to adjust the power of the photostimulation. In order to shield the light during active use, the lasers, drivers, wheels, mirror, and the collimators were positioned inside a custom-made closed box made of black cardboard (TB4, Thor lab, Germany). Using SMA connectors, a dual fiber-optical coupler chord (≅4m long; core diameter: 200µm, numerical aperture: 0.37) was connected to the collimators and the other end of the cord could be connected to the implanted cannula of a freely moving animal. Finally, this setup was connected to an Arduino with a custom-made script (written by Horst Obenhaus and modified by Ida V. Rautio) for providing pulsating photostimulation (pulse frequency of 60Hz). The cord was suspended over the observer chamber along with the stimulation cord, attaching both of them together with small pieces of tape to avoid entanglement while the animal was moving around.

2.3.1 Fiber optic cannula testing

The cannulas used for implantation were individually selected by testing the intensity range and spread of the light from the tip of the etched fibers before surgery. Each cannula had two optic fibers (1 mm diameter between them) that could be individually controlled by the two lasers. In order to calibrate both these fibers individually, the light intensity at the fiber tips was measured using an optical power and energy meter (PM100D, Thor Lab, Germany). When both the fibers were emitting 30mW (+/- 2mW) by adjusting the dimmer wheels, the patch cord was disengaged from the cannula and the intensity of both the lasers at the identical specifications were recorded. The values on the dimmer wheel, intensity of both the fibers of the cannula, and the light intensity without optical fibers attached - for both left and right lasers - were noted and used to calibrate the lasers before each experiment to the correct light intensity that was recorded for each optical fiber. Both the fibers were separately calibrated such that emission from each of them would deliver the light at an intensity of 30mW (+/- 2mW) to both hemispheres when attached to the patch cord, and the recorded light intensity without optic fibers was used as a proxy for the correct light intensity during the experimental sessions.

2.4 The task

The script used to automate the experimental protocols and record the performance metrics of the animals was designed by Benjamin A Dunn and modified by Ida V. Rautio. These were written in Python and were run using Python 2. The experimental protocols could be run in either manual mode or automated mode. In manual mode, the experimenter had control over the light of each of the stimulus balls (SB1 and/or SB2) - choosing if and when to turn them on and off independently - and it was within the experimenter's control to stimulate the animals as well. This mode was used for training the performer animals before the actual experiments.

In automated mode, the script ran automatic *trials* where SB1 and SB2 would light up in a set sequence for a maximum amount of time of 30 seconds unless pushed by the animal. The steps for one *trial* (Figure 2.2) were as follows; **Step 1:** Once the script started to run, SB1 would light up after a time delay of between 3-30s and would then remain lit for a maximum of 30s. If the ball was not pushed within the 30s, the light would turn off and the trial was counted as a *missed trial*. **Step 2:** If SB1 was pushed within 30s, SB1 light would turn off and SB2 would light up for a new maximum period of 30s. If SB2 was not pushed before the 30s, the trial was stopped and counted as a *failed trial*. **Step 3:** To get the rewarding stimulus, the animal had to push SB2 within the 30s, and if this happened the trial was counted as a *successful trial*. When the light was turned off at any point during any of these steps (either by failing to hit the ball within 30s or by completing the trial successfully) a new trial would initiate with a time delay of 3-30s.

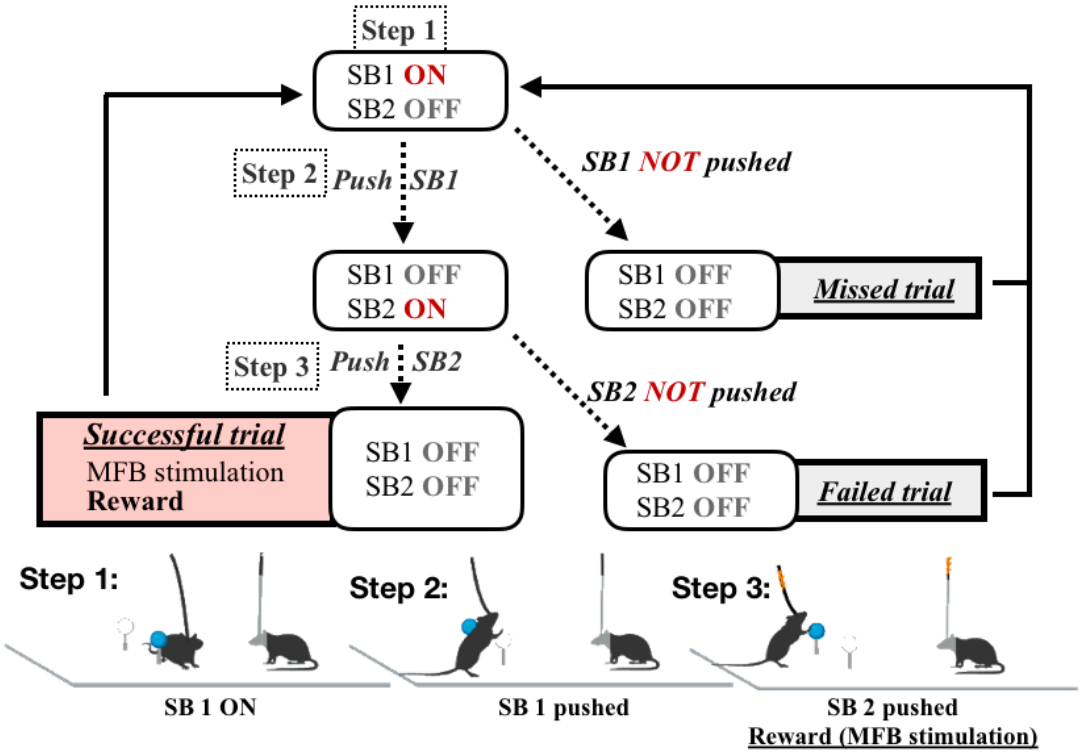

Figure 2.2: Visual representation of successful *trials, failed trails,* and *missed trials.* The dotted arrows represent choices presented to the animal. Reward is provided to both animals when the task is executed (step 3).

This means that to get a successful trial and receive rewarding stimulation, the animal had to first push SB1 within 30s of it lighting up, and then push SB2 within an additional 30s. Each 30-minute *session* contained multiple trials, and how many potential trials became available for the animal depended on the performance of the animal itself. The number of simulations and trials was also recorded for later use in the analysis.

The script used for the control condition for the experiment controlled *trials* where the SBs would be turned on in the same sequence as previously described (SB1 turning on, then off before SB2 turns on and then off) for a random period of time each (but still in the same sequence, SB1, and then SB2) to imitate the sequence of a successful trial, also delivering a rewarding stimulation when the light in SB2 turned off. This was independent of whether they were pushed by the naïve animal moving around in the chamber, as the light and reward delivery was completely controlled by the Raspberry Pi. Thus, a trial was initiated when SB1 lit up, which happened anytime between 3 and 30 seconds after the script was run or after a trial had finished. Then, SB1 would turn off randomly between 3 and 30s, irrespective of whether the animal was pushing the ball or not, after which SB2 would light up. SB2 would then randomly turn off (3-30s) resulting in the delivery of a rewarding stimulus to both animals. This control condition was designed to dissociate the task-specific behavior of the performer from the rewarding stimulation, such that we could better determine if it was the behavior of the animal doing the task that was important for the observational experimental condition.

2.5 Training of model rats

Some animals were trained to perform the behavioral task beforehand, since performers were needed for the observational paradigm. These animals were either previous observers or a naïve animal, trained manually by the experimenter by way of different shaping techniques and utilizing the previously described script in manual mode (section

2.4). The length of the training differed between animals, but one was considered a performer when they could do the task for 30 mins for a period of 4 consecutive days, with the proportion of successful trials as ≥ 75% in all sessions.

2.6 Surgical procedures

2.6.1 Electrode implantation

Habituated animals at the minimum age of 12 weeks and weight 400 grams underwent electrode implantation (n = 22) (Figure 2.3). The rat was anesthetized using 5% isoflurane (0.6 L/min) and transferred to a stereotaxic frame when loss of consciousness had been established (David KOPF, USA). When under anesthesia, Marcain (25 mg/mL, intradermal, near incision site), Metacam (2 mg/mL, intramuscular) and Temgesic (0.3 mg/ml, intramuscular) were administered, after which the flow of isoflurane was maintained at 0.3-0.4 L/min and the concentration kept between 1.5-3%. Before implantation, three to four stainless steel screws were attached to the skull using a drill. These were intended to anchor the implant in place. The rat was then implanted with a stainless-steel bipolar stimulating electrode at the right MFB (AP: -2.8, ML: +1.7, DV: +8). The drilled hole was closed using a medium-viscosity silicone adhesive (Kwik-sil, USA). Once this hardened, the implant and screws were treated with self-cure dental adhesive resin cement (Super Bond, Sun medicals, Japan). After this dried, dental acrylic cement was used to secure the implant. The cementing was checked for sharp edges and the wound was sutured if needed. Details of method mentioned in Appendix A, supplemental protocol 1b and equipment and manufacturer details in Appendix B. The implanted animals were given Temgesic (0.3 mg/ml, intramuscular) 6-12 hours after surgery and Metacam (2 mg/mL, intramuscular) 24 hours after surgery. A minimum of 5 days were given for recovery, before used for experimentation.

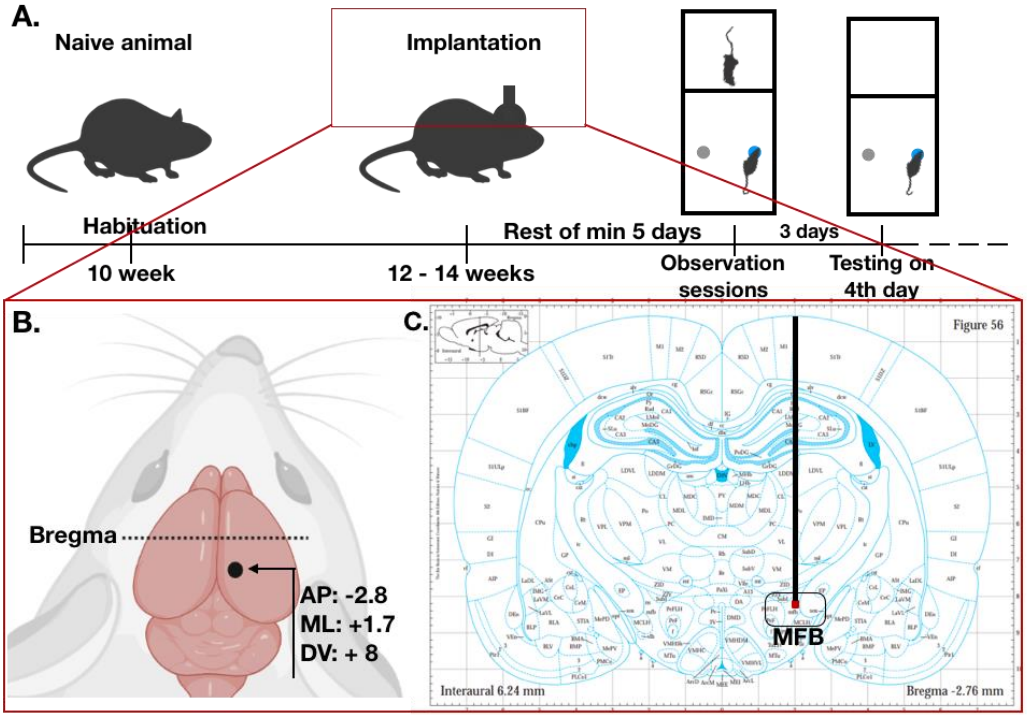

Figure 2.3: **A.** Timeline of electrode implantation. **B.** Schematic view of implant location. **C.** Coronal plane view of the implantation site illustrating the stimulating electrode (black line), map obtained from brain atlas (Paxinos and Watson, 2006).

2.6.2 Viral injection, optic fiber and electrode implantation

At 9 weeks, the selected animals were used for viral injection which is followed by an electrode and fiber implant surgery, 2-3 weeks after viral injection (Figure 2.4). The rat was anesthetized, transferred to the stereotaxic frame, and medicated. The viral vector used (AAV5-mDIx-Chr2-mCherry-Fishell-3) was made at the Viral Vector Core Facility at Kavli Institute for Systems Neuroscience (Trondheim, Norway). The virus (700nl) was lightly colored with fast blue and centrifuged to mix. The solution was transferred to two glass micropipettes that were prepared in house using a micropipette puller (Sutter Instrument, USA). The solution was backfilled with mineral oil. The virus was injected bilaterally (700 nL in both hemispheres) targeting the ACC (AP: + 2.5, ML: ±0.5, DV +2) with a flow rate of 50 nL/m that was achieved using a microinjector pump (World precision Inc, USA). To avoid backflow, withdrawal of the micropipette was done after letting the micropipette rest for 10 mins after injection.

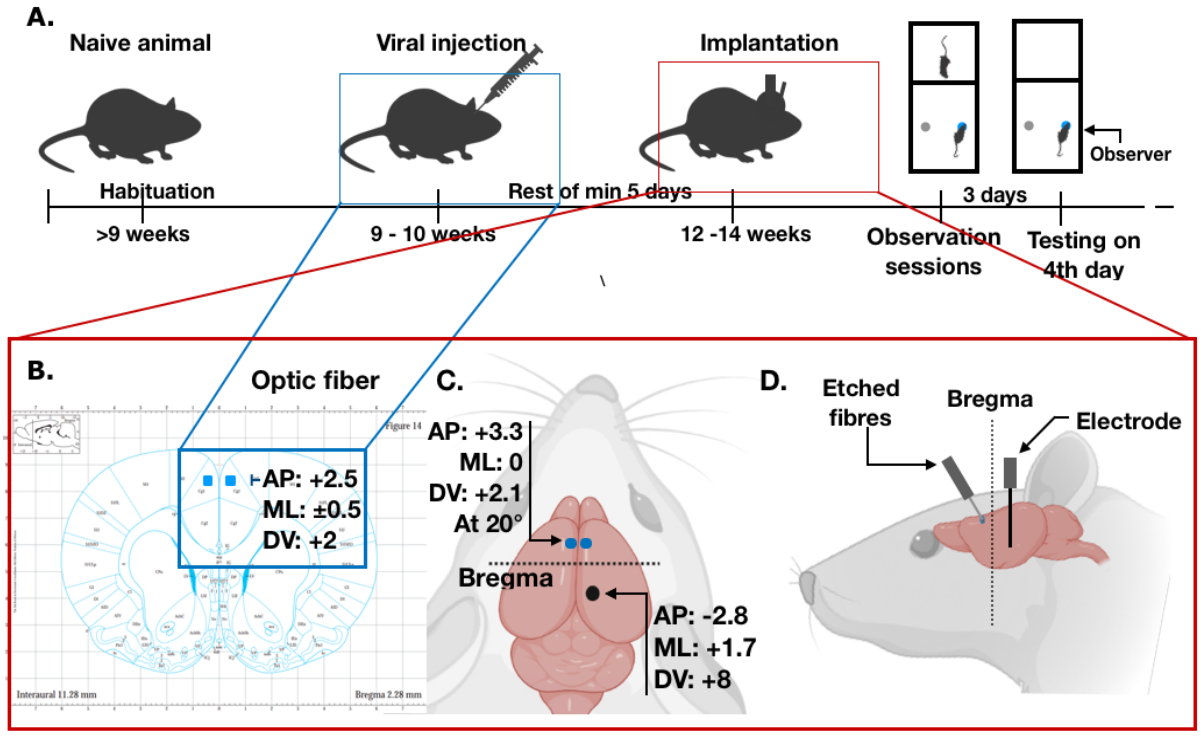

Figure 2.4: **A.** Timeline of electrode implantation. **B.** Coronal plane view of implant location at +2.28 AP coordinate from Rat brain atlas (Paxinos and Watson, 2006). **C.** Schematic view of the implantation site, depicting viral injection (blue) and fiber implant (black). **D.** Side view of the implantation site illustrating electrode and fibre implant.

Once the injection was complete, the cranial hole was sealed using Kwik-sil and the incision was sutured closed. After 2-4 weeks and when the weight of the animal was a minimum of 400 grams, the rat underwent a second surgery for fiber-optic and electrode implantation. The fiber-optic cannula was purchased from Doric lenses (Canada) and etched with 48% hydrofluoric acid at SINTEF (Trondheim, Norway) under the supervision of Ida V Rautio. Each cannula was calibrated prior to the surgery and their ID number, intensity and corresponding raw intensity was noted before implantation (see section 2.3.1). After the incision site was opened, a bipolar stimulating electrode (AP: -2.8, ML: +1.7, DV: +8) (P1 technologies, Canada) and then the cannula (AP: +3.3, ML: 0, DV: +2.1, at 20°) (Doric lenses, Canada) was implanted. The craniotomy was closed using medium-viscosity Kwik-sil. Securing of the implants, cementing, and post-operative procedures were similar to that as mentioned in section 2.6.1. A detailed procedure for

surgeries and virus injection is mentioned in Appendix A, supplemental protocol 1a and 1c, and equipment and manufacturer details in Appendix B.

2.7 Box habituation three days before experimentation

To establish a baseline behavior in the box, the animals were habituated to the observational chamber from the age of 10 weeks. This was done such that the animals were comfortable in the box for a minimum period of 30 mins in a low-light and silent environment. After surgery the animals were left to recover for one day before re-habituating them to confirm that they were still comfortable in the experimental box. Three days before starting the experiments, a two-day protocol for experiment specific habituation was initiated, followed by testing of the implanted electrode on the third day. On day one the observers were placed in the performer chamber for an untethered 30-minute session. On day two, they were placed in the performer chamber for a tethered 30-minute session. If the observers were comfortable (like, relaxed ears, grooming, laying down, or bruxing) and did not show signs of stress (like, freezing, perked up ears, or puffed hair), they were used for the experiments. It was ensured that observers were not exposed to the performer chamber outside of these days, thus all habituation sessions prior to surgery were confined to the observer chamber.

2.8 Electrode and fiber testing one day before experimentation

The fibers were tested prior to implantation (section 2.3). On the electrode testing day (one day before the experiment began), the script used for the experimental sessions (detailed in section 2.4) was used to manually stimulate the animals. To test the effect of the stimulation, a neutral object was placed at the center of the observation chamber. Initially, single stimulation (of lower strength, 18-22mA; strength adjusted using ISO-Flex stimulation box (A.M.P.I., USA)) was provided each time the animal looked at or approached the object. If the animal showed signs of aversion towards the object (like, moving away from the pen, vocalized pain, or freezing), the electrode was considered ineffective. Additionally, an electrode was considered ineffective if there were motor defects observed even though signs of aversion were absent (i.e., involuntary motor movements). If the animal was interested in the object without any motor defects, the strength of stimulation was increased until the animal showed strong interaction towards the object (the maximum strength varied between animals, 18-30mA). When the interaction with the object was strong, multiple bursts of electrode were provided (3-4 bursts for 2 seconds). The electrode was considered a working electrode if the animal showed clear signs of experiencing a rewarding effect from the stimulation without any motor defects or aversion to the neutral object (i.e., strong interaction with the object; like biting the object or carrying the object in clockwise motion). When testing the electrode of a fiber-optic-implanted animal the effect of the electrode was tested in an identical way as described above, but in addition we considered potential effects of concurrent optogenetic inhibition. If the testing of the electrode showed positive effects, the lasers for the optogenetic inhibition would be turned on and the animal would again get stimulating while interacting with the pen or being in close proximity to it. This was to make certain that the rewarding effects of the stimulation were also present during optogenetic inhibition, while at the same time controlling for any potential side effects from the inhibition itself irrespective of any experimental conditions.

2.9 Behavioral experiments

All three experimental protocols were performed across the span of four days: three days of observation followed by a testing day to test whether the observer had learned the task

or not. During the first three days the observer was placed in the observation chamber followed by the performer in the performer chamber. The 30-minute sessions would begin when both animals showed signs of comfort (like, relaxed ears, grooming, laying down, or bruxing, section 1.3). Once the script started to run, the elapsed time was followed closely using a timer, and the animals were undisturbed for the entire period of the experimental session. The observer rat was allowed to freely move in its environment while the performer rat would perform the task for the next 30 minutes (there was no trained performer rat in the control group; further detailed in Table 2.2). Both observer and performer would receive stimulation when the performer successfully performed the task and thus completed a trial. Once the 30 minutes were over, the performer was removed and placed back into its home cage, before the observer was removed from the observer chamber and placed in his own separate home cage. On the fourth day, the observer was placed in the performer chamber alone and the script controlling the experiment was initiated and left running for 30 minutes and the behavior of the observer rat was recorded both in a text-file and by video recording. There were 3 experimental groups: observational experiment, control experiment, and observational experiment with ACC inhibition (Table 2.1 and 2.2).

	Day 1 to 3 (Observation days; 30 min)				Day 4 (testing day; 30 min)	
	Performer		Observer		Observer	
	Trained	Stimulation	Trained	Stimulation	Trained	Stimulation
Animal placed in	**Performer chamber**		**Observer chamber**		**Performer chamber**	
Observational experiment	Yes	Self	No	Via performers Performance	—	Self
Control experiment	No	Script	No	Script	—	Self
Observational experiment with ACC inhibition	Yes	Self	No	Via performers Performance	—	Self

Table 2.2: Technicalities of the experiments. Observational experiment included a trained performer and a naïve observer, both stimulated when the performer completed a task in automated mode (section 2.4); Control experiment included a naïve performer animal and a naïve observer, both stimulated using control experiment script (section 2.4). That is, during the first three days, both the naïve animals received stimulation independent of their behaviors inside their respective chambers since the script controlled the stimulation; Observational experiment with ACC inhibition included a trained performer and a naïve observer, both stimulated when the performer completed a task in automated mode (section 2.4). However, unlike the observational group and control group, the observers of this group had their ACC region ontogenetically inhibited through the use of optic fibers during all three 30 min sessions. The ACC was not inactivated during testing day (Table 2.1).

2.10 Tissue handling and specimen preparation

Upon completion of the study, the animals were anesthetized with Isoflurane (0.8 L/min) and injected with a lethal dose of pentobarbital (400 mg/mL, Exagon 50 mg/Kg) administered intraperitoneally. Prior to euthanasia, a peristaltic pump with two outlets was prepped. The first outlet released freshly made ringer solution (0.85% NaCl, 0.025% KCl, 0.02% NaHCO3, pH 6.9), while the second outlet directed 4% PFA (diluted from 200mL freshly made 10% PFA with 0.4 M PBS buffer, pH 7.4) (Appendix A, Supplementary protocol 1.d). Once the rats stopped responding to mechanical stimulation intended to check for pain reflexes, transcranial perfusion was carried out. Incisions along either side of the chest were made to open the thoracic cavity and expose the heart. Perfusion was

initiated by inserting a G1/2" needle into the left ventricle of the heart allowing the ringer solution to start entering the body, quickly followed by a small cut of the right atrium, allowing the blood to flow out of the body. After approximately 200mL of Ringer had been circulated (or the blood had turned colorless), the Ringer solution was exchanged for PFA. Once the fixation tremors usually observed when PFA entered circulation was finished, the needle was removed, and the animal was decapitated. Skin and muscle were then removed from the skull which, along with the implant(s) still attached, were refrigerated overnight in 4% PFA. The following day the skull was carefully opened to allow for the brain to be removed and to allow the implants to be carefully extracted from the brain before it was refrigerated in 2% DMSO for cryoprotection until further use.

2.10.1 NISSL staining

The stored brains were sliced using a sliding microtome (Thermo scientific HM430, USA). The cryoprotected brain was sectioned into 40 µm slices onto a cleaned superfrost plus slides (Thermo scientific, menzrl-glaser, USA) and collected into three series that were collected in protective tubes. Series 1 was used for slide preparation while series 2 and 3 were stored in a deep freezer for further studies or as backup. Using Tris buffer, the sliced tissue was carefully arranged onto the superfrost slides such that slices were evenly spaced for scanning. These slides were left for drying in a covered space overnight at room temperature. The tissues were then stained using the Nissl staining protocol (detailed in Appendix A, Supplementary protocol 2). After overnight drying, the slides were cleaned, and excess wax was removed. The slides were then scanned using an automated slide scanner (Zeiss Axioscan Z1, Germany). The images were processed using Zen Blue software.

2.10.2 IHC staining

While slicing, the cryoprotected tissues were both fixed on a slide and carefully collected into two series. One of these series was used for immunostaining using goat anti rat IgG (1:1000 dilution, incubated overnight) (H+L Alexa fluor 546, ThermoFisher, USA) and RFP antibody (1:1000 dilution, incubated for 1hr) (chromotek, Germany) (Protocol 3; Appendix A), and were transferred onto superfrost plus slides for scanning. The slides were detected at 546 nm and scanned using Zeiss automated slide scanner, Germany.

2.11 Data quantification

Observational learning was tested on the testing day (day 4), by placing the observer in the performer chamber. After a 30 min long session, latencies, successful trial count, and trial speed of the observer was calculated using a custom-made python script written by Ida V. Rautio. For this thesis, ***Trial speed:*** successful trials completed by animal per minute (stimulations/session length); ***Average trial length:*** average time taken by animal to complete one trial, ***Performance:*** proportion of successful trials out of total possible trials, and ***Latency***: average time stimulus ball (SB) 1 or 2 was ON. Apart from these numbers, learning was validated with specific behavioral traits exhibited by the observer on testing day. This included a ***sequential pushing*** pattern where the observer would exhibit understanding of the task by systematically pushing the SB1 first and then pushing SB2 to receive the reward. This behavior was observed in the form of ***toggling***: where the observer moves from SB1 to SB2 in order to receive the reward when the SB1 turns on. Every trained performer exhibited toggling during the performance, therefore, an observer that has learned the task would exhibit toggling behavior as well. This behavior is illustrated in Supplementary Figure C8 (Appendix C).

2.11.1 Software for Quantifying Rodent Attentiveness

The recordings from each experiment were first processed using a custom-made software called "Rodent Attentiveness Quantifier" (RAQ), which was designed by Michael S. Larsen as part of a bachelor's project for the course TDAT3022 at Norwegian University of Science and Technology (Protocol 3, Appendix A). The software was designed based on a deep learning body pose estimation software (DeepLabCut 2.2b8, DLC; Kim *et al.,* 2020, Clemensson *et al.,* 2020), and was tailored to process the 30-minute-long videos into shorter sequences with head angles (described below) and frame numbers displayed on the video frame (Figure 2.5). This was done in three steps. Step 1 included cropping the video to confine the analysis to the chambers of interest. In step 2, the DLC trained model would assign a vector to the observer's head using the ears and snout as reference points. A second vector was assigned to the chosen point of interest, which could be the performer and/or stimulus ball (Figure 2.5, A). In step 3, the head angles were calculated as the angle between the observer's head vector and the performer (i.e., performer-referenced), or between the observer's head and the stimulus ball (i.e., stimulus-ball-referenced). The head angles and corresponding frame numbers were displayed in the processed videos (see examples in Figure 2.5 B and C).

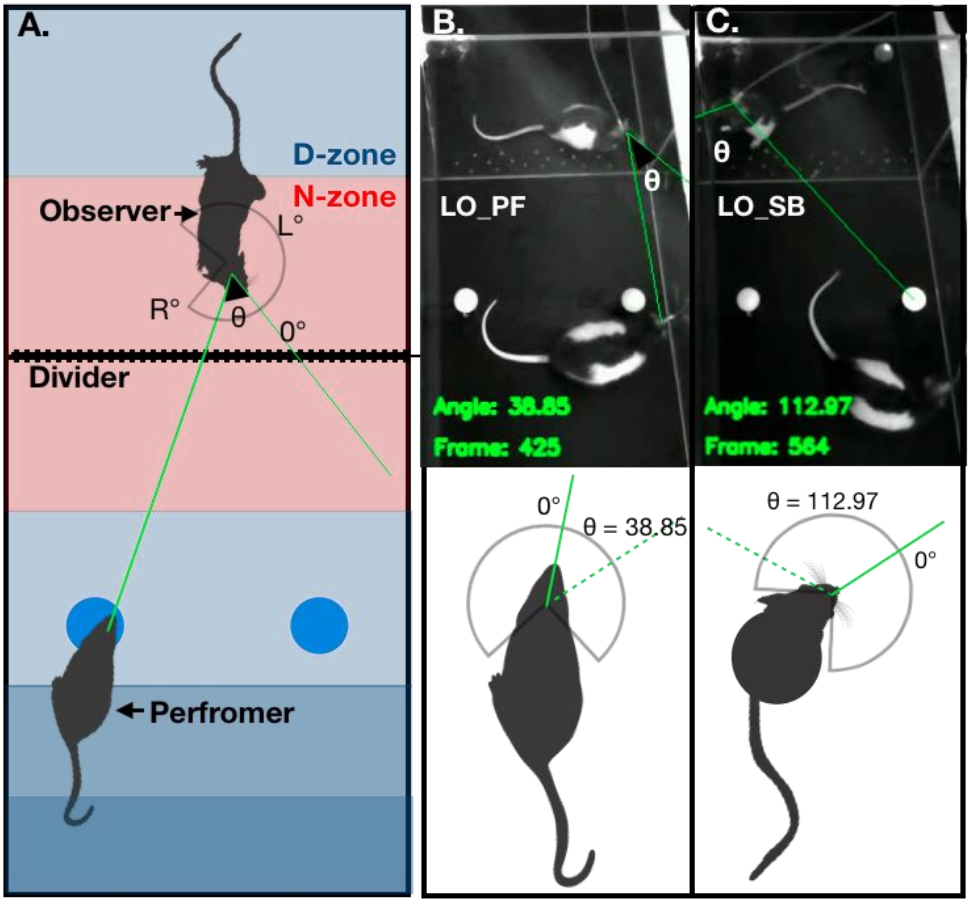

Figure 2.5: **A.** Calculation of head angle using RAQ. Angle θ represents the angle between the snout of the observer (at 0°) to the snout of the performer. **B.** Example of one frame from a light-on **performer-referenced** (LO_PF) processed video when the observer is attentive (A) to the performer. **C.** Example of one frame from a light-on **stimulus-ball-referenced** (LO_SB) processed video when the observer is partially inattentive (PI).

In a manual analysis conducted in parallel, the head angle was matched to the direction of the observer's head and called *head direction*, and this was used to estimate which visual field (left or right, L/R) the point of interest (performer or stimulus ball) was occupying. Manual annotation of animal behavior was noted using this value (represented as L/R θ). The resulting data included the processed video, comma-separated variable (CSV)-files of the animal's position in the box and head angles. The RAQ software, however, was unable to process head angles during certain behaviors (for example, when the animals were interacting; see Appendix C, Supplementary Figure C5).

2.11.2 Protocol testing

The attentiveness of the observer during the observation days (first three days) was studied via frame-by-frame analysis of the processed video recordings, and manual labeling of the behavior. The RAQ software could analyze the videos in multiple ways, hence it was important to decide on one form of processing for both software-based (henceforth called *automated analysis*) and manual analyses. To decide which method was feasible to study attentiveness, one random video was selected from the observational experiment group, control experiment group, and observational experiment with ACC inhibition group. These three videos were used to compare all forms of analysis produced by RAQ software that were of interest to the author, manually score the behavior (Figure 2.6 and Table 2.3), and then decide which protocol was optimal. Comparisons were done between 30 min videos (all frames; AF) to check observers' attentiveness towards stimulus ball (AF_SB) and performer (AF_PF). 2. Additionally, processed videos only contained frames where SB1/2 lights were ON (task relevant timestamps; LO), with angle inputs between observer and stimulus ball (LO_SB) and/or the performer's head (LO_PF). Examples of light-on processed video are demonstrated in Figure 2.5 B and C.

2.11.2.1 For automated analysis

The CSV files produced by these processed videos where run through a custom-made MATLAB script (Protocol 4, Appendix A) which classified head angles in each frame into two categories of attentiveness, *attentive* (≤40°), *possibly attentive* (40° to 110°), and two categories of inattentiveness, *possibly inattentive* (110° to 140°), *inattentive* (140° to 176°). The total percentage of time spent in these four categories was noted for comparison while distribution of the head angles was visualized using a histogram.

2.11.2.2 For manual analysis

The same videos from these tests were also used for manual labelling of behavior through frame-by-frame analysis using head angle, head direction, animal behavior, and frame numbers. For the purpose of this project, only a set of behaviors were focused on during analysis, including exploring (walking and jogging), resting (sitting or laying down), social interaction (sniffing, fighting or rearing against the divider when the performer was close by), and grooming. These actions were further categorized based on the position, head angle, and head direction of the animal (Table 2.3). It was noticed during preliminary testing that animals did not move rapidly within frames, such that analysis of one frame at a time would be an effective way to score behavior. Therefore, the movement of the animal (every 200 frames) was grouped into one of four classes of attentiveness: attentive, possibly attentive, possibly inattentive, inattentive. Figure 2.6 shows the range of head angles considered for these groupings that are color coded as; attentive (turquoise), possible attentive (green), possible inattentive (orange) and inattentive (pink). The percentage of these four categories of attentiveness and behavior of the observer was noted for comparison.

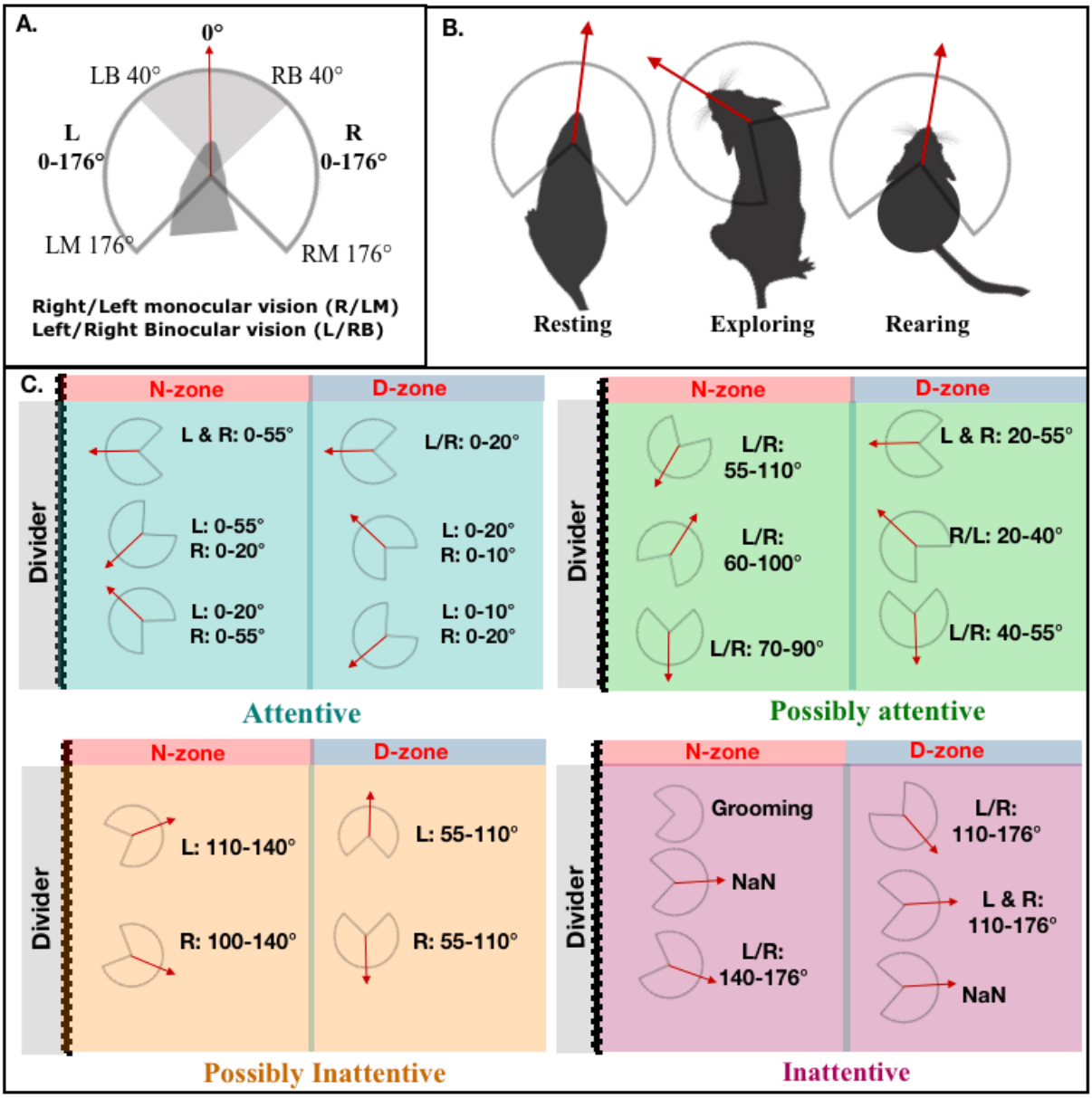

Figure 2.6: Database of head angels of the observer at different attentiveness states. **A.** Schematic representation of the rats' field of vision with the area spanning from center to left monocular vision represented as left vision (L=0-176°) and the area spanning from center to right monocular vision represented as right vision and (R=0-176°). Binocular vision is represented in grey. **B.** Examples of the mentioned field of vision on rats while resting, exploring, and rearing **C.** The database of possible head angles and head direction for analyzing behavior of the animal in the chamber with regards to the performer, which in this schematic would always be positioned to the left of the divider. Here, left (L; the range only specifies for left field of vision), right (R; the range only specifies for right field of vision), or left and right (L & R; the range is the same for both directions) shows the possible head directions.

Assumption	Position	Range	Observed action
Attentive* (A)	N zone	R/L 0-110°	**Exploring:** approaching the divider or performer, rearing against the divider/walls of N zone **Resting:** standing or laying while snout directed towards the bulbs or performer **Social Interaction:** mimicking the performer, interaction at the divider [includes rearing against the divider/sniffing with performer]
	D zone	R/L 0-70°	
Possibly attentive* (PA)	N zone	R/L 70-130°	**Exploring:** approaching the divider or the walls, rearing against the walls of N/D zone **Resting:** standing or laying while snout directed towards the performer chamber/N or D zone walls at given angles **Social Interaction:** interaction at the divider [includes sniffing, rearing against the divider with/without performer]
	D zone	R/L 20-110°	
Possibly Inattentive** (PI)	N zone	R/L 50-160°	**Exploring:** approaching N zone or walls of N/D zone, rearing against the walls of N/D zone at said angles, toggling between N and D zone **Resting:** standing or laying within specified angle **Interactions**: no social interactions, interaction at divider [includes sniffing, rearing against the divider] when performer is not near by
	D zone	R/L 90-150°	
Inattentive** (IA)	N zone	R/L 100-176° Or NaN	**Exploring:** moving in D zone, walking away from N zone **Resting:** standing or laying down while snout is turned to given angles **Interaction:** no social interactions, interacting with walls of N/D zone, rearing when at given angles. **Grooming**: always considered as inattentive behaviour.
	D zone	R/L 80-176° Or NaN	

Attentiveness = A + PA; **Inattentiveness = PI + IA

Table 2.3: Shows the behavioral classifications drawn from the position, head angle and action of the observer. The groups are coded with specific colors ***attentive*** (turquoise), ***possibly attentive*** (green), ***possibly inattentive*** (orange), and ***inattentive*** (pink)

2.11.3 Manual analysis of attentiveness

After comparing various analysis protocols, recordings of observation days (first three days of the experiment) of all groups were further processed using RAQ to include only the timestamps when stimulus balls were lit. Since the focus of this thesis is to check for social information processing, manual analysis was conducted using frames where SB lights were ON (task relevant timestamps), with angle inputs between observer and performer (LO_PF). These videos were typically 8-12 min in length and displayed the head angles and frame number. The attentiveness of the observer for these processed videos was studied by manual frame-by-frame analysis as mentioned in 2.11.2.2.

2.11.4 Statistics

Results are expressed as mean±SD for attention scores and performance, and sum±SD for latencies, successful trial count, and trial speed (explained in the start of section 2.11). Time spent by the performer performing the task was calculated using length of processed videos (LO_PF). The head angle plots, bar graphs, and scatter plots were programmed in MATLAB (R2021a 9.10.0), while pie charts and line graphs were made in iOS numbers (10.12.6). The Bland Altman plot and all statistical analyses were performed using SPSS version 27.0.1.0. A descriptive test was first done to test for normality of the data. Since the data had many outliers and the number of datapoints were not enough to determine normality, nonparametric alternative tests were used to evaluate animals' performance, trial speed, trial count, and average trial length. Additionally, the SD (standard deviation) of every observed parameter for this set of data was very high, suggesting a wide distribution. Distribution of attention scores were evaluated using the SPSS normality test

(Shapiro-Wilk and Kolmogorov Smirnov test), then statistical comparisons were made using nonparametric tests (Table 2.4). Statistical significance, set at p-value <0.05, was tested between variables mentioned in Table 2.4. Correlation studies were done using Spearman's rank-order test (rho).

Variable	Normality test	Within groups	Between two groups	Between all groups
Testing day scores of observer	Shapiro-Wilk test	——	Mann–Whitney U test	Pairwise Kruskal–Wallis test
Performance of performer		Signed-rank test		
Attention		Signed-rank test or one-sample Kolmogorov-Smirnov		

Table 2.4: Shows the statistical tests done on collected data using SPSS 27.0.1.0. First, group description is done to check for shrewdness of the data. This along with a normality test determined whether to use nonparametric alternate tests. Mann–Whitney U test was done for two-independent samples or groups (number of successful trials executed by observer on testing day; latencies of observer for stimulus ball 1&2 on testing day; trial speed of observer on testing day). Pairwise Kruskal–Wallis test of k-independent samples was done to check for distribution of various variables across all groups (sum of number of successful trials executed by performer over 3 days; mean attentiveness scores of observers over 3 days; mean trial speed between groups and performer)

Henceforth, animals (observers or performers) from observational experiments will be termed as **observational group;** control experiment animals as **control group**; and animals from observational experiments with ACC inhibition as **ACC-inhibited group.**

Section 3

Results

3.1 Histology

After the experiments were finished, each animal was perfused, the brain was sectioned and NISSL stained (Appendix A, Supplementary protocol 2). Placement of the MFB electrodes were validated based on the animals' behavior as well as post-hoc inspection of the tissue against the Rat Brain Atlas (Paxinos & Watson 2007). As shown in Figure 3.1 A, a trace left behind by the tip of the bipolar stimulating electrode was located near the MFB at -2.76mm (AP) from bregma, in this specific example. A similar method was used to locate fiber optic cannula implant traces, which could be observed at +2.28 AP from bregma in the specific example in Figure 3.1 B.

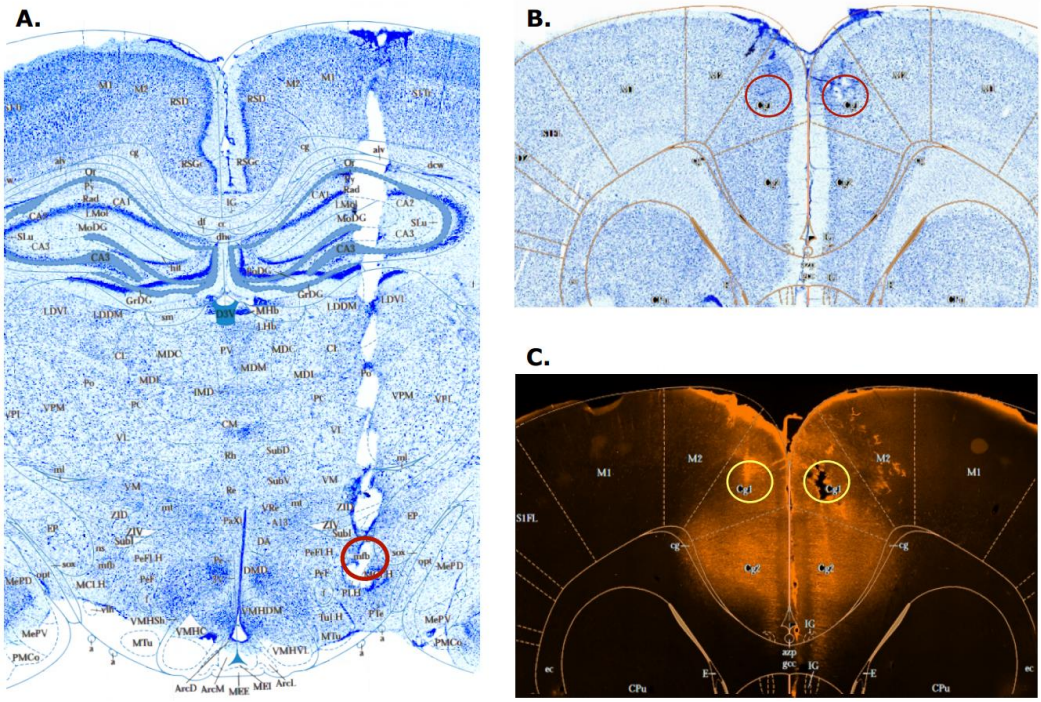

Figure 3.1 Shows, **A**: NISSL scan of electrode implant overlaid with an image from the Paxinos & Watson Rat brain atlas at -2.76 AP. The MFB region is highlighted in a red circle; **B:** NISSL scan of fiber optic implant at Cg1/Cg2 overlapped with rat brain atlas coordinates of +2.28 AP; **C**: Immunohistochemistry of viral expression at Cg1/Cg2 overlapped with rat brain atlas coordinates of +2.28 AP. Overlay made by Ella H. Holmberg using Adobe Illustrator and coordinates from Rat Brain Atlas by Paxinos & Watson (2006).

Virus expression was visualized using fluorescent immunohistochemical staining against the mCherry protein, visualized under 546 nm light, after which the slides were scanned using an automated slide scanner (see section 2.10). Using the Paxinos & Watson Rat Brain Atlas (2006), we confirmed the virus expression was confined to Cg1 and Cg2 (Figure 3.1 C). In a few cases, slight spread of the virus was observed into parts of the motor cortex (M2) (ID 27323 and 27322; see supplementary Figure C3, Appendix C). No motor artifacts were exhibited by these animals. All brain slices scanned for electrode

implantation, fiber optic implantation, and virus expression showed that the targets were hit as intended (Appendix C, Supplementary Figure C1, C2, and C3).

3.2 Social information promotes learning

Learning was measured as the proportion of successful trials out of the total number of trials (henceforth called "performance"), latency to press the correct ball when lit, and trial speed. Additionally, behaviors attributed to the task (toggling and pushing) were checked (mentioned in section 2.11). All animals from the observational group performed the task on the testing day. However, their performance varied from 2.7% to 60% (SD=22.81, mean=30.87). Performance data was considered a continuum, and learning was interpreted based on group comparisons. Four out of six observers from the control group were classified as non-learners (mean performance=0%), while two animals had a performance rate of 13.2% and 15.4% respectively (control group SD=7.42). On testing day, four observers from the observational group exhibited both toggling and strategical pushing of the stimulus ball 1 and 2 (explained in Supplementary Figure C8, Appendix C). The extreme values (2.7% and 7.7% performance) however, were considered as non-learners, due to the lack of toggling behavior. Their movements matched that as illustrated in Supplementary Figure C8 in Appendix A. Similarly, the observers from the control group did not show any signs of toggling.

Observers from the observational group showed higher performance of the task on average (30.87%, n=6), which was six times higher than control observers' average performance (4.77%, n=6). Additionally, the average trial speed of observers from the observational group was significantly higher (U=4, p=0.02, mean=0.4 successful trial/min) than the control group (mean=0.06 successful trial/min). Mann–Whitney U test showed that these two groups were significantly different from each other in performance and speed (U=4, p=0.026). The latency of observers to tap SB1 did not differ across groups (U=8, p=0.132), however, the latency of control observers to tap SB2 after SB1 was significantly larger (U=3, p=0.015) compared to the observational group (16.83 latency). An overview of these results is given in Table 3.1.

	Day 1 to 3			Day 4 (testing day; 30 min)			
	PST% of performer/script			Observer data (Day 4)			
	Day 1	Day 2	Day 3	PST (%)	TS (St/SnL)	ATL (s)	SB2.L (s)
Observational experiment	98.17 ±3.45	90.37 ±11.59	94.15 ±12.92	30.87 ±22.81	0.40 ±0.3	40.85 ±8.04	16.83 ±4.82
Control experiment (untrained performer)	100.0 ±0.00	100.0 ±0.00	100.0 ±0.00	4.77 ±7.42	0.06 ±0.09	54.99 ±6.65	26.73 ±5.24
Observational experiment with ACC inhibition (without outlier)	99.16 ±1.88	99.30 ±1.06	94.26 ±7.37	0.65 ±11.21	0.08 ±5.73	58.41 ±8.02	29.69 ±0.18
Observational experiment with ACC inhibition				18.48 ±39.88	0.27 ±0.43	49.92 ±19.09	24.60 ±11.39

Table 3.1: Mean results of all performers and observers in each of the three groups. PST: Proportion of Successful Trials (performance, %); TS: Trial Speed (Stimulations/Session Length); ATL: Average Trial Length; SB2.L: Latency to tap Stimulus Ball 2 after first tapping Stimulus Ball 1.

3.3 ACC inhibition disrupts observational learning

In the ACC inhibited group, four animals were considered definitive non learners (performance = 0%), while two performed the task at 2.6% and 89.8% (see Table 3.1). The one animal with a high performance, however, exhibited hyper-locomotive behavior when tested, continually lapping the perimeter of the arena, and hitting SB1 and SB2, as opposed to loitering and toggling between SB1 and SB2 in a directed manner. On average, observers with ACC inhibited performed worse (18.48%; n=5; SD=39.88) than observers from the observational group (30.87%; n=6), however the difference was not statistically strong when outlier data was included (U=6, p=0.126; outlier data: observer 27260). Without the outlier data, the average performance of the ACC-inhibited group was significantly weaker than the observational group (U=0, p=0.01), with an average performance of 0.65% (n=4; SD=11.21). The raw data is presented in Appendix C, Supplementary data Table C1.

ACC inhibition also decreased the trial speed of the animals (n=6; Mean=0.27 successful trial/min, SD=0.43). When the outlier was removed (n=5; Mean=0.08 successful trial/min, SD=0.18), the difference in trial speed between observational group and ACC inhibited group becomes significantly larger (U=0, p=0.038). Similar results were observed for the average trial length, where the ACC inhibited observers took a longer time to complete the trials on average (n=4; mean ATL=58.41±8.02) as shown in Figure 3.2. Latencies of ACC inhibited observers were also reduced, but again, a significant difference was revealed when the outlier was removed (SB1: U=2, p=0.038; SB2: U=0, p=0.01). In contrast to control group, average latency (towards SB1) of observers with inhibited ACC was statistically different from that of the observational group, when the outlier was removed (U=8, p=0.038). Interestingly, we noted during the analysis that the outlier animal appeared to mimic the performer's actions at a continually increasing rate during the three-day observational learning session (day 1:15% mimicking, day 2:57.12%, day 3:62%). This has been discussed further in section 4.7.1.

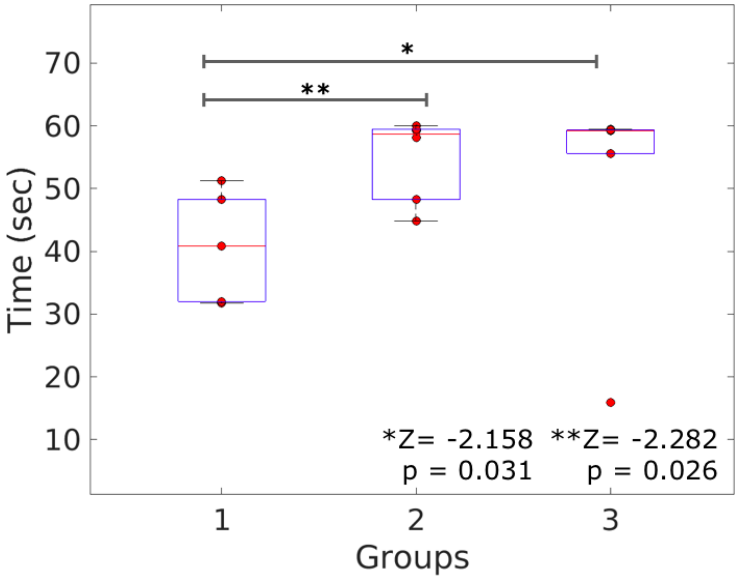

Figure 3.2: Average trial length (ATL) of observers from the observational group (1, n=6); control group (2, n=6); and ACC inhibited group (3, n=5). Red dots represent one observer, while the red line indicates the median. Upper and lower extremes are represented with black whiskers, and the 25% and 75% quartiles are represented by the upper and lower blue boxes, respectively. The Z-scores and p-values of the across-group comparisons are shown at the bottom right. Figure made in MATLAB R2021a.

3.4 Performance of the performers were consistent across groups

Trained animals were used as performers only after they passed the learning criteria of ≥ 75% in multiple training sessions. The control group script that was used to stimulate the animals was designed to match the performance of a live animal. A pairwise Kruskal Wallis test was performed for all three groups as independent samples. The performance of

performers across the observational group and ACC inhibited group was statistically similar ($p>0.05$). The groups were also similar for the performers' speed and total number of successful trials over the 3 days ($p>0.05$). The control group was significantly different from both the observational group ($p=0.015$), and the ACC inhibited group ($p=0.038$) in performance. An overview of these results is given in Table 3.1. Spearman's test showed no significant relation between performance of performer and observers' performance on testing day ($rho=0.276$, $p=0.412$). However, a slight non-significant positive correlation was seen between the performance of observer and performer in the observational and ACC inhibited group ($rho=0.28$, Supplementary Table C8 C, Appendix C). Consequently, time spent by the performer to do the task was negatively correlated to the observer's average trial length.

3.5 Attentiveness scores were better estimated with manual analysis

Attentiveness of the observers towards the performers was quantified using a custom-made attentiveness scoring system, where the observers' head directions and body language during observational sessions were divided into two categories: attentiveness (subdivisions: Attentive (A) and Partially Attentive (PA)) and inattentiveness (subdivisions: Partially Inattentive (PI), and Inattentive (IA)). The values were represented as a percentage of time spent by the observer in each of these categories (example given in Supplementary Figure C4, Appendix C). After collecting data in protocol testing, and both automated and manual analysis, the first step was to check for normality. The Shapiro Wilk test showed that A, PA, and I categories of all groups (over three days of observation) were not normally distributed ($p>0.05$). Hence, significance in these analyses was determined using non-parametric statistical tests.

3.5.1 Protocol testing

To establish the most effective analysis method to quantify attention, three protocols were tested using three random videos (as mentioned in 2.11.1). Comparisons of the attentiveness score of the raw videos were made between the automated (CSV file of head angles) and manual analysis (behavior analysis of observers in processed videos with head angles superimposed). The results showed that attentiveness scores were similarly distributed across three categories (A, PA, and IA; $p>0.1$, Kruskal Wallis test). To isolate task-relevant social information, the raw videos were successfully processed using RAQ to get light-ON processed videos (LO, that included extracted frames where stimulus balls were on). A comparison of attention scores between LO automated analysis and LO manual analysis, reported significant similarities between experiment groups ($p>0.1$, Mann–Whitney U test). Similar results were also achieved when comparing distributions of attention scores between automated analysis of lights-on performer-focus videos (LO_PF), and lights-on stimulus-ball-focus (LO_SB) videos ($p>0.1$, Mann–Whitney U test). Within attentiveness (A and PA), we observed a general trend that partially attentive (PA) scores from the automated analysis were larger than manual analysis scores. Similarly, partially inattentive (PI) scores were not equally distributed between manual and automated ($p<0.05$, Kruskal Wallis test) (Supplementary Table C2, Appendix C). Due to these differences and certain shortcomings in the automated analysis (discussed in 4.8), every recording was processed using lights-on performer-focus (LO_PF), after which automated and manual analyses were done. RAQ software was also used to plot positions of the animals in the box. These scatter plots clearly show the toggling movement of a trained performer and naive observer (explained further in Supplementary Figure C6 A and C, Appendix C), as compared to the random movements of naive performers in the control group (Supplementary Figure C6 B, Appendix C).

The output of the automated analysis (head angle CSV file) was analyzed in MATLAB and visualized with the head angles binned in histograms (Figure 3.3A). The angles between observer and performer during task execution trials are expressed in degrees. The bins were grouped into four overarching categories of attention. The percentage of bin counts were calculated and then compared against output from the manual analysis.

3.5.2 Automated analysis vs manual analysis

According to both automated and manual analyses, the observers were attentive >60% of the time across all three days. However, there was a difference in mean attentiveness scores (A+PA) between the automated (80.23%) and manual analysis (64.50%) (raw data presented in Appendix C, Supplementary Table C4 and C6). We tested the normality of the distributions of automated analyses with a Mann-Whitney test, which showed there were significant difference in all four categories of attention (U=23, p=0.00). While investigating the reasons for such a difference, it was noted that the automated analysis left some head angles uncalculated (NaN angles; see MATLAB code in Appendix A, Supplementary protocol 5). The percentage of NaN angles in the automatically processed videos ranged from 7% to 37%. The higher ranges of uncalculated angles were considered as information loss and, hence, the manual mode of analysis using shortened videos was selected as the better method of quantifying attention of the observers toward task execution. Nevertheless, the RAQ software aided in filtering out irrelevant frames from the videos for analysis, leaving less manual work to be done.

		Observation group					ACC inhibited group				
		1.	**2.**	**3.**	**4.**	**5.**	**1.**	**2.**	**3.**	**4.**	**5.**
1.	Manual_A (%)	—					—				
2.	Manual_IA (%)	-1.00*	—				-1.00*	—			
3.	Automated_A (%)	-0.09	0.09	—			-0.56	0.56	—		
4.	Automated_IA (%)	0.09	-0.09	-1.00*	—		0.56	-0.56	-1.00*	—	
5.	PF_Performance (PST; %)	-0.43	0.43	-0.71	0.71	—	-0.36	0.36	0.00	0.00	—
6.	OB_Performance (PST; %)	0.20	-0.20	-0.54	0.54	0.55	0.23	-0.23	-0.45	0.45	0.78

**Correlation is significant at the 0.01 level (2-tailed Spearman's rho)*

Table 3.2: Shows correlation coefficients between attention scores and performance of both performer (PF) and observer (OB) from the observational group and ACC inhibited group. Performance of performers and attention scores were averaged over three-day sessions (data shown in Supplementary Table C4 and C6), while performance of the observers was taken from the testing day (summarized in Table C1). In this case, A: Attentiveness (A+PA); IA: Inattentiveness (I+PI); PST: Proportion of Successful Trials (performance, %); Significance p <0.01.

A Spearman's correlation test did not reveal a significant correlation between the manual and automated analyses (see Appendix C, Supplementary Table C7). However, we found that attentiveness of the observers tends to be correlated positively with their performance in all three experimental groups when analyzed manually (rho= 0.10). This pattern was reversed in the automated analysis, with attentiveness of the observer tending to correlate negatively with performance of the observer (rho= -0.27). Interestingly, both

types of analyses suggested a negative correlation between performance of the performer and attentiveness of observer, though these trends were non-significant.

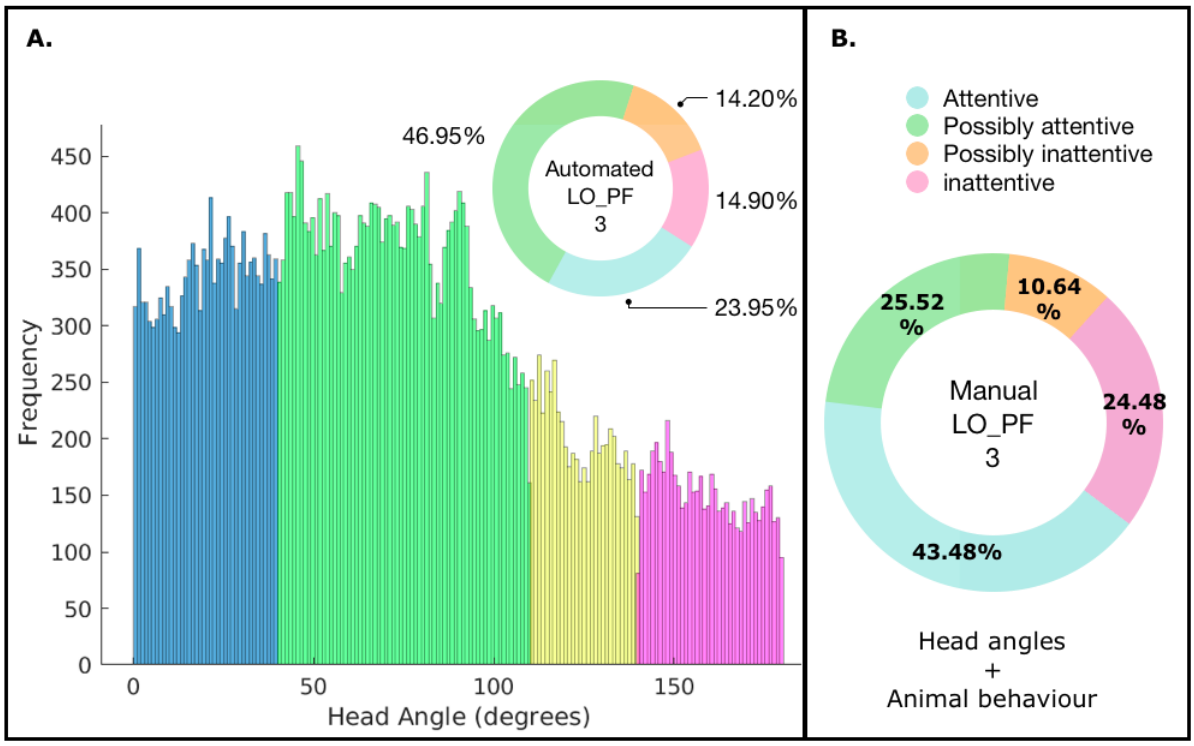

Figure 3.3: Comparison between automated and manual analyses of attention of a lights-on performer-focused (LO_PF) processed video from ACC inhibited group. **A**. Histogram shows the bin count (or frequency) of degrees ranging from 0-176 (based on RAQ software). The degrees represent the angle between observer and performer during task execution. The bins are grouped into four categories of attention. Inserted image is a pie chart representation of the attention scores from the histogram. **B.** Manually calculated attention scores of the observer from the same LO_PF video.

To further assess the differences in calculated attentiveness seen in the observers in all groups, a Bland–Altman plot was used. In it, the attention scores were compared between manual and automated analyses by finding their mean, difference, and then plotting Bland-Altman (Figure 3.4). As expected, the data was randomly distributed with no observable trend. Additionally, the figure shows that for both attentiveness and inattentiveness, most of the data points fell outside the 95% confidence level (limits of agreement). Hence, the data from the automated analysis method was considered different from manual scoring.

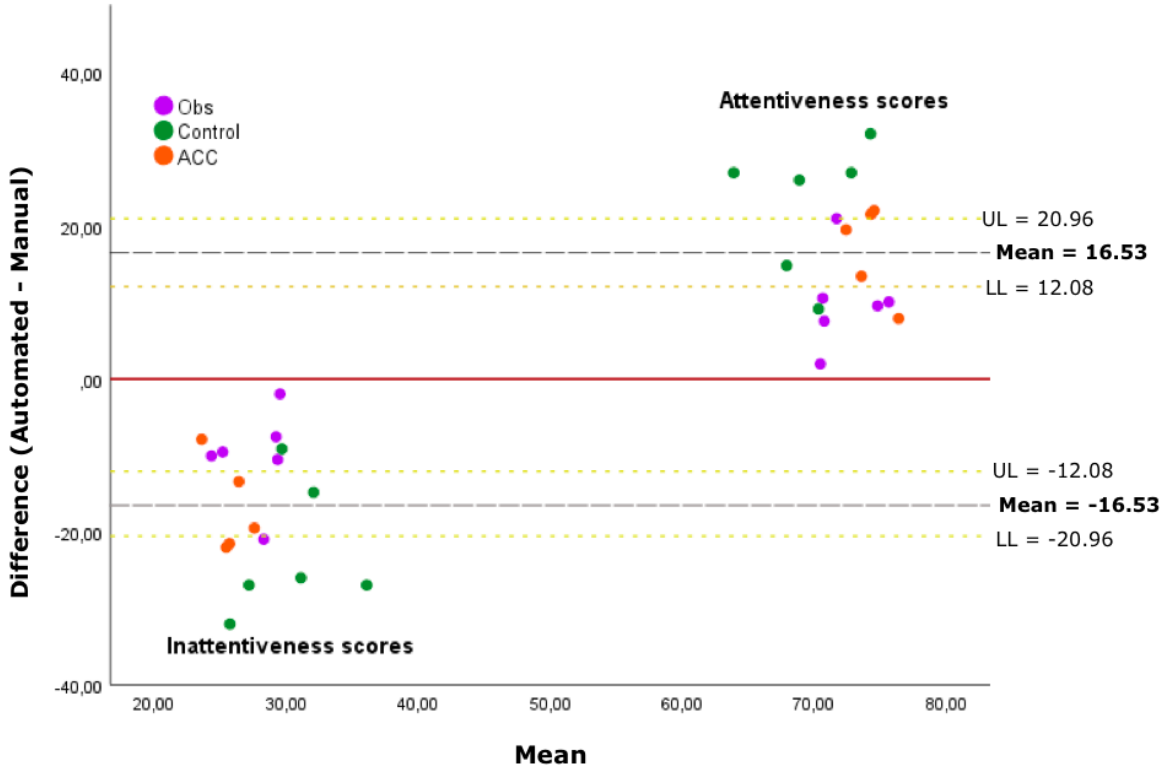

Figure 3.4: Bland-altman plot of attentiveness (A+PA) and Inattentive (PI+I) score, comparing automated and manual analysis. The scores were calculated using lights-on performer-focused (LO_PF) processed videos from observational group (Obs), control group (Control), and ACC inhibited group (ACC). Red line: origin; black dashed line: mean of both groups; yellow dotted line: lower and upper limit of the 95% confidence interval for the average difference (agreement); mean= [(attentiveness score manual+attentiveness score automated)/2]; difference= (automated-manual).

3.6 Attentiveness of the observer was consistent across groups

The processed videos were analyzed using head angle data (achieved with RAQ software) and manual scoring of behavior (section 2.11.3). The result suggested that the observers were attentive 58-68% of the time in all groups. A Shapiro-Wilk test confirmed that the data was not normality distributed ($p>0.05$). Mann Whitney and pairwise Kruskal Wallis tests both showed that the attention of the observers was equally distributed across groups ($p<0.05$). The mean attentiveness score of observers in the observational group was 67.30%±3.58, while the control group scored 58.37%±5.09. Observers with ACC inhibition scored 65.83%±4.04. However, those with ACC inhibition scored slightly higher (mean score 53.79%) in the Attentive (A) category compared to the Observational group (mean score 48.53%) and control group (mean score 42.26%), with a standard deviation of ±6.33. In contrast, the mean attention scores of observers in all other categories were similar (SD; PA=±2.1; PI=0.26; I=1.71). The mean attention scores for each of the groups are illustrated in Figure 3.5. A Spearman's correlation test did not reveal significant correlations between the performance and attentiveness of the observers in any of the groups. However, as mentioned in the previous section, there was a trend towards positive correlations (Table 3.2). Figure 3.6 shows the similarities in attentiveness of observers, while illustrating the relationship between performance of performer over all three experimental sessions and observer performance on test day. It additionally highlights the extreme values of both observational group (*) and ACC group (**).

Finally, we assessed the attentiveness of the observers across the first three days and found a significant positive correlation between day 1 and day 3 ($p<0.01$), indicating that the observers were not equally attentive towards the performer during the three days of observational session. Conversely, a significantly negative correlation was observed between inattentiveness of the observer on day 1 and day 3 (Appendix C, Supplementary Table C8 A).

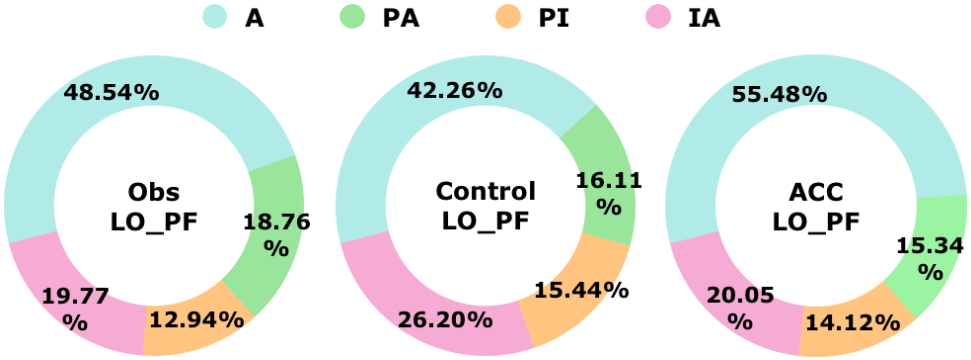

Figure 3.5: Shows comparisons between lights-on performer-focused (LO_PF) processed video from observational group (Obs), control group (Control), and ACC inhibited group (ACC). Attention score gives the percentage of time spent by the observer being attentive (A, blue); partially attentive (PA, green); partially inattentive (PI, yellow); and inattentive (IA, green).

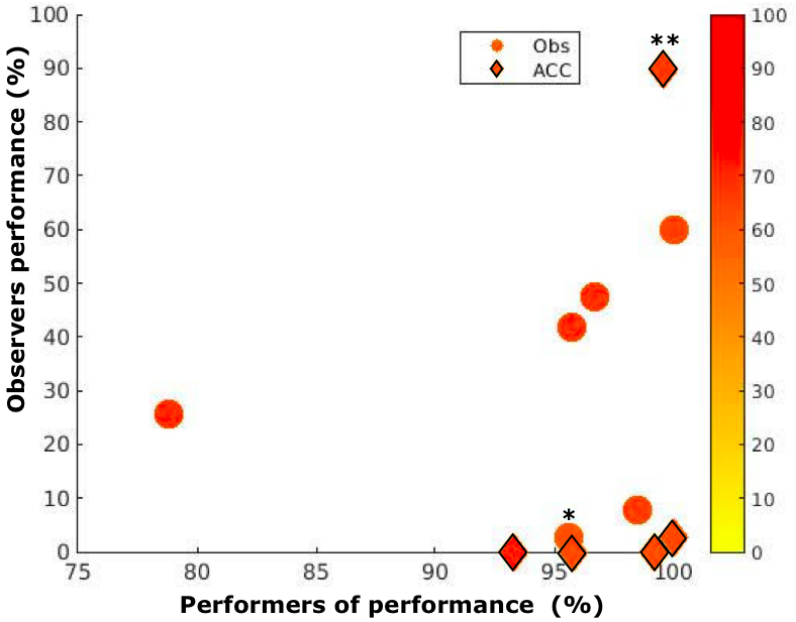

Figure 3.6:

Scatter plot shows correlation between performance of performer (averaged over three days) and performance of observer on testing day. The color bar (z-axis) represents attention scores of respective observers averaged over three days. Single star (*): observer 27171 (performance: 2.7%, attention score: 61.23); double star (**): observer (performance: 89.9%, attention score: 66.85). Control group not included because of automated script controlling the task (That is, performance of performer is 100% in all cases).

Section 4

Discussion

4.1 Result summary

Previous conditioning studies on rodents have investigated the possible role of attention in learning (Bryden *et al.,* 2011, Allsop *et al.,* 2018, Carrillo *et al.,* 2019, Schneider*et al.,* 2020). The goal of the current study was to investigate how social information or inhibition of the ACC impacts attention during observational learning. The data collected in this study show that social information (i.e., an active demonstrator) is critical for observational learning, since observers in control groups did show signs of learning after three days of exposure to the task with naïve animals as performers (mean performance=4.77%, SD=7.42). By comparison, the group which observed active demonstrators performed the task at significantly higher rates (mean performance=30.87%, SD=22.81, U=4, p=0.022). When the ACC of observers was ontogenetically inhibited the performance of the animals decreased on testing day (mean performance=18.48, SD=30.87). Outside of a single outlier, the animals showed no signs of learning the task. A Spearman's correlation test further showed that performance of the observers correlated positively with the amount of time the performer spent executing the task (rho=0.651, p=0.03). The attention of the observers during three days of observation sessions was quantified by manually scoring the animals' behavior using head angle data calculated using custom-made software, the Rodent Attention Quantifier (RAQ). The results showed that, on average, observer animals paid attention to the performer 60-70% of the time across all groups. Thus, inhibition of the ACC did not impact the attention of the observer animals. We did not see a significant correlation between the observers' performance and their rate of attention. Quantification of attention using only the data from RAQ software was also attempted (automated analysis), and the resulting data was significantly different from manual analysis (U=23, p=0.00). No significant correlation was found between performance of performer and attention of the observers in any group.

4.2 Methodological considerations

One of the key objectives of this thesis was to find an effective method to analyze the behavior of the observers in the observational learning paradigm. Data was collected by three experimenters (described in section 2.2). The protocols for MFB stimulation and ACC inhibition were established by Ida V. Rautio, while methods used for Nissl staining and immunohistochemistry were developed beforehand by researchers at the Kavli Institute for Systems Neuroscience. The data used for this study is part of a larger ongoing PhD project investigating the role of the ACC during reward-motivated observational learning. To this end, additional data for all three experimental groups are still being collected.

Previous studies have shown that animals can learn through visual, somatosensory, olfactory, and auditory cues (Nicol 1995, Bryden *et al.,* 2011, Schneider *et al.,* 2020). Additionally, differences have been reported for the neural mechanisms of social learning between females and males (Burgos-Robles *et al.,* 2019). However, detailed analysis of factors like sex and sensory cues responsible for learning were beyond the scope of the current study. Hence, we used only male Long Evans rats that were habituated to the apparatus and experimenters before the start of experiments, being careful to keep the experimental surroundings as consistent as possible through all experiments and all conditions (section 2.2 and 2.7).

Performance of the performers and the attention scores for observers were calculated over three days of observation sessions. However, performance of the observer was tested on the 4th day, and hence only a single datapoint was recorded per observer. Since these three variables needed to be compared to one another, performance of performers and the attention of the observers was averaged across the three days. The raw data for this calculation is presented in section 3.2 (Table 3.1) and Appendix C (Supplementary Table C4 and C6). Unless otherwise specified, these data were used for statistical analysis and correlation studies detailed in this thesis.

4.3 Histology

As seen in the results (section 3.1), the tip of the electrode hit the MFB bundle in all animals, suggesting successful reward-stimulations. However, it is important to note that the histology of the implantations only validate the observed behavior of the animal during electrode testing (section 2.8). The deciding factor for the use of implanted animals for experimentation was the testing day. Stereotaxic coordinates for MFB implants are not absolute, since MFB extends from nucleus accumbens to ventral tegmental area (Figure 1.4 A). Other studies have used AP coordinate -2.52 mm to get a successful reward stimulation (Dandekar *et al.,* 2019). Hence, an animal was established as having a working electrode only after demonstrating stimulation-induced behavioral reward and reinforcement in a separate test session after the observational learning task was concluded (section 2.8).

4.4 Using reward as motivation to learn

It has been demonstrated in previous literature that rodents can learn through observation to avoid aversive cues or to receive food reward. In both of these cases, motivation to learn was fear (Allsop *et al.,* 2018, Carrillo *et al.,* 2019) or hunger (Carlier & Jamon 2006, Jurado-Parras *et al.,* 2012, Schneider *et al.,* 2020). Similar methods have been used to study depression and addiction in rodents (Olds 1958, Negus & Miller 2014, Dandekar *et al.*, 2019). A study from 2007 on reward motivation showed that rats with electrode implants in the MBF would perform the lever-pressing task significantly faster than the baseline group (Carlezon & Chartoff 2007). This was because they would self-stimulate themselves as reward, while the baseline group received food reward. They concluded that intracranial self-stimulation (ICSS) of the MFB can be used as an effective tool to motivate animals to perform several tasks. Therefore, the current work tool an alternative approach to study observational learning in rats by providing MFB stimulation as the reinforcer. As explained by Bandura (2008) and Nabavi (2012), the 'internal need to learn' is one of the key factors for social learning. Therefore, the author credits using this form of intangible reward to being a step forward in understanding a complicated behavior like observational learning.

4.5 Instrumental role of social information in observational learning

Learning in our paradigm was assessed by the correct performance, trial speed, and average trial length of the observer on the testing day. Additionally, behavior of the observer on the testing day was used to further validate learning (section 2.11). Based on the results, it was very clear that the animals indeed learn through observation when rewarded by MFB stimulation. As expected, the control group did not learn the task after three days of observation. For this group, the task was controlled by a custom-made script as described in section 2.4. The naive animals received stimulation each time the script ran the task. If the animals had merely associated the visual cue (lights of stimulus balls) to the reward and learn the task, the control animals would have shown higher

performance rates on testing day with toggling behavior. However, that was not the case, suggesting that the goal-directed behavior of a trained demonstrator provides critical social information for the eventual performance rates seen in the observers.

As explained in the introduction, attention is an important factor in social learning, and it has been shown to be important for observational learning in rodents (Allsop *et al.,* 2018, Carrillo *et al.,* 2019). It was therefore important to quantify the attention of the observers in these experiments. Hence, an analysis method was formulated to measure attention and generate quantitative scores both manually and using automated analysis.

4.6 Quantification of attention

The RAQ software was used to process 30 min observation sessions (days 1 to 3) such that the frame number and head angles between the observer and performer (or stimulus ball) was calculated (2.11.1). After testing various protocols (2.11.2) and manually scoring the resulting videos, it was noticed that attentiveness (A+AP) was consistent across groups. Hence, it was concluded that light-on performer-focused (LO_PF) processed videos were the most ideal form of analysis for this thesis. Other reasons for this decision were that the LO frames were relevant to the task and focusing on the head direction of the observer with respect to the performer would be more useful to study the acquisition of social information. Any interactions outside these frames were considered non-relevant social information.

After manual and automated scoring (MATLAB script described in 2.11.2) using processed videos, it was concluded that attention was consistent across both observational and control groups. By ruling out attention as a distinguishing factor between groups, the data strongly suggest that the critical factor to learn the task was the actual demonstration of the task by a well-trained performer. Figure 3.6 shows that the attention of the observers did not play a critical role in the observers' performance, since animals that performed the worst did not necessarily pay less attention to the performer (marked by the single star).

4.7 ACC inhibition disrupts observational learning, but not attention to task-specific information

Previous studies demonstrated that the ACC also has a significant role in fear-based observational learning (Jeon *et al.,* 2010, Allsop *et al.,* 2018, Keum *et al.,* 2018, Carrillo *et al.,* 2019). Our study therefore took a different path and explored a form of observational learning which was reward motivated. Based on its involvement in fear learning, it was hypothesized that inhibition of the ACC would also affect observational learning in our paradigm. Preliminary results show that ACC inhibition blocked observational learning in most of the rats in our study. This result ties in well with earlier studies that have demonstrated the involvement of ACC in both food reward and punishment for social learning (e.g., Schneider *et al.,* 2020). The current results, however, go beyond previous reports by suggesting an involvement of the ACC in observational learning when reward is not physically tangible (internal need to learn, as discussed in 4.4).

As expected, attentiveness (A+PA) scores showed that observers with their ACC inhibited paid equal attention to the performer. Studies have speculated that activity of ACC was related to the attention of the animal (Bryden *et al.,* 2011, Schneider *et al.,* 2020). However, it is important to note that the concepts set forth previously on the neural mechanisms of attention specifically talk about the role of ACC in attention towards a target (or targeted detection) (Posner and Petersen 1990). Bryden and colleagues did not

work on a social experiment, rather an odor recognition task where the animal had to learn the task through trial and error (Bryden *et al.,* 2011). Hence, their reports on attention cannot be directly compared to the present results. Schneider et al., reported that the persistent activity of ACC during observational tasks, was connected to the attention paid by the animal to the auditory cue and outcome prediction (Schneider *et al.,* 2020). Again, in our experiments we considered the attention of the animal toward another animal rather than an auditory or olfactory cue. To the knowledge of this author, the role of ACC on attention for such a paradigm has not been conducted before. In our case, disruption of attention due to an inhibited ACC was not expected, since the attention system of the rat brain was not close to the inhibition site (discussed below in 4.7.1). As expected, the current work demonstrates that at least the social attention of the animals was not affected by ACC inhibition.

One variable other than attention and social cues that could affect learning of observers in the ACC inhibited group was the performance of the performer. To exclude weak performance by the performer as a potential reason for non-learners, a statistical test was done to check similarities in performance across groups (see section 3.4). The results showed that the performance of the demonstrator animals did not differ between the ACC inhibited group and observational group over the three days of training. Hence, the current data support the ACC as processing social information in rats. Interestingly, it was noticed that correlation studies suggested a slight negative correlation between average performance of performer and observer (Supplementary Table C8 B, Appendix C). However, upon further investigation, this relationship was evident only when control group data was included in the correlation studies. The correlation between performance of the performers and observers was positive but not significant when calculated for the intact observers and the ACC-inhibited group (Supplementary Table C8 C, Appendix C).

4.7.1 An extreme outlier in the data set

An unexpected observation was that animals with ACC inhibition had a higher performance rate on testing day (performance: 89.48%) when compared to the control group (Figure 3.5). Upon further inspection, this was attributed to a single outlier in the group. Observer #27260 demonstrated signs of learning during testing day, by toggling between the stimulus ball for the reward. A fascinating fact noticed while analyzing his recordings was that he demonstrated mimicking behavior at an increasing rate during the three days of observation (section 3.3). Although imitation is not the only reason for learning, studies have shown this to be a powerful key to learning behavior (Wallace 1870, Kawamura 1959, Tomasello *et al.,* 1987). It was also noted that the animal tended to stay closer to the divider and watch the performer most of the time during task performance. This could be visualized in the position plot made with the data points provided by the RAQ software (Supplementary Figure C6 D, Appendix C). To investigate further, the raw videos of this interaction were studied. It was noticed that the animal had a high activity level throughout the sessions. Whereas most observers would groom and explore the D-zone frequently, this observer did not. It was previously shown that rat ACC contains mirror neurons that respond to the first-hand experience or observation of pain, as demonstrated by Carrillo *et al.* (2019). However, Carrillo and colleagues recorded their cells at AP: +0.96mm, ML: +0.3mm, DV: -3mm, while our inhibition was at AP: +2.5mm, ML: ±0.5mm, DV: +2mm. Even though our virus expression was spread across a certain region of the ACC, it is completely possible that mirror-like neurons at more anterior locations were unaffected by the virus. The region used for recording by Carrillo and colleagues has also been associated with attention systems (van Heukelum *et al.,* 2020). Another possibility was that the animal did not have a properly inhibited ACC, which was ruled out since his Immunohistochemistry scans showed good viral expression (Supplementary Figure C3, Appendix C). However, this behavior was not observed in other animals with inhibition, so

until more data is collected and analyzed these ideas remain speculative, and the data point is considered an outlier.

4.8 Automated analysis of attention is not completely reliable

Past studies have attempted to understand different behavior analysis methods by comparing automated and manual analysis or human annotation (Rousseau *et al.,* 2000, Lorbach *et al.,* 2018). While these experiments showed that automated analysis was a reasonably acceptable substitute for manual scoring, they focused on identifying direct social-interaction behavior in rodents. In this study, attention was measured with behavioral changes of the animal during task performance. This was done by using the observer head angle information calculated by the RAQ software and scoring attentiveness by: automated analysis (using MATLAB script, section 2.11.2.1) and manual scoring of the behavior (section 2.11.2.2). Few experiments have used software's to control for attention, specifically, by calculating differences in the orientation of the animals' body with respect to the target (Allsop *et al.,* 2018, Carrillo *et al.,* 2019). However, they have not emphasized on validating or explaining these automated results, leaving room for speculation on their validity.

For the data used in this thesis, the attention scores produced by both these analysis methods were consistent across groups. However, Spearman's correlation analysis suggested a negative correlation between automated analysis attention scores and performance of the observer (Table 3.2). What this would have meant, is that the more the observer paid attention to the performer, the less it learned the task. This was curious, since some of these observers were already established to be learners (from the observational group). The attention scores, therefore, were not explaining the learning behavior of the observer. Further probing brought to light that in most videos, up to 30% of the head angle data was calculated as NaN (as explained in 3.5.2; examples given in Supplementary Figure C5 B and C, Appendix C). Furthermore, another way information was lost while using automated analysis was incorrectly calculated angles, as seen in Supplementary Figure C5 A and D (Appendix C). It was noticed that this happened due to the bird-view position of the camera. If the camera was recording from over-head position, the RAQ software could have shown less errors, since there would be clearer visibility of the animals' movements near the D-zone and corners (e.g., Rousseau *et al.,* 2000, Lorbach *et al.,* 2018). However, placing an overhead camera would not assist in studying the behavior of the observer, since visibility of the observer's face was more in the bird-view position of the camera (like the one used for this project). These uncalculated or incorrectly calculated frames were scored by the author during manual analysis, so information was not lost in that case. For the automated analysis, however, the behavior of the observers during these lost time stamps were not considered for analysis, which could explain the difference in results produced by manual and automated analysis (as illustrated in the Bland Altman plot; Figure 3.4).

Moreover, a common trend observed in automated analysis was that partially attentive (PA) scores were very high in all data points (Supplementary Figure C7, Appendix C). Upon further analysis, it was speculated that this was caused due to the movement of the performer along the horizontal axis of the observer's visual field. For example, when the observer was in N-zone and looking towards the performer chamber, the performer toggles and does the task as trained. Let us consider that in this scenario, the observer does not move its head. The calculated angle still differed because of the performer's movements. Previous documents have recorded that rats can independently rotate their eyes (Wallace *et al.,* 2013). So, it is not completely correct to say that the observer was not paying attention or was paying less attention, since theoretically, the observer could have watched the performer by gliding its eye horizontally without moving its head. These

minor details were noted better with human-annotation. Hence, manual analysis was considered the preferred form of attention scoring.

4.9 Manual scoring could assist in understanding social interaction

As noted just above, manual analysis was the more reliable form of attentiveness scoring, and they showed a positive correlation between attentiveness the performance of the observers on testing day. An interesting observation was that the performance of pre-trained performers showed a slight negative correlation to the observers' attention in both observation and ACC inhibited groups (rho<-0.35; Table 3.2). This could be explained with the time spent by the performer interacting with the observer during task performance (distraction). It would be interesting to see if this trend sustains or becomes significant for a larger data set, since it could further connect the attention of the observer to some aspect of observational learning. For example, it was noticed that each time a performer executed a trial immediately after interacting with the observer near the divider, the observer would pay closer attention to the performer performing the task. This was a common trend observed in both observation and ACC inhibited groups.

Additional analysis was done to test differences in attention of the observer between the three days. It was interesting to see a significant positive correlation between day 1 and day 3, but not day 2 (Supplementary Table C8 A, Appendix C), and that this trend was observed in all groups. The author speculates that this trend would average out in a larger data set, since according to the Mann Whitney test, attention was the same across all groups. Therefore, with additional data, it is possible that the differences between attentiveness of observers between days could be reduced further.

4.10 Shortcomings

Sample size: Most of the experiments and observations made in this thesis were based on small data sets that also contained outliers in each group (Supplementary Table C1, Appendix C), which could skew mean values and introduce variation. Hence, the low sampling size could have influenced statistical conclusions. However, the objectives were achieved with the given data, and the results clearly demonstrate the potential use of attention analysis demonstrated in this thesis. Additionally, as mentioned before, more behavioral data continues to be collected for all of the experimental groups.

Influence of the animals' behavior on results: During habituation we also noted individual differences in the behavior between animals, and this could have affected results in many ways. For example, the learning demonstrated to some degree by the outlier (section 4.6.1) was attributed to imitation. However, it could be completely possible that the animals had an alert and hyperactive personality by nature. Removing such data points is not a solution since variations like these are to be expected in humans as well. Therefore, to study observational learning under a realistic condition, data points like these need to be understood better rather than being removed. The best step to take, is to dilute these differences with additional data points.

Potential experimental bias: A common problem associated with behavioral studies, is bias from experimenters, especially on a complex behavior like attention (Zentall 2012, Nabavi 2012). Even though the current project attempts to remove potential human bias to a great degree, complete unbiased analysis of attention cannot be achieved. It has been demonstrated in the results that automated analysis has significant shortcomings to be trusted completely for a complicated procedure like attention scoring. However, combining automated analysis to make manual scoring more precise is an effective way to move

forward, until other computational tools are invented. This has been demonstrated in this thesis.

Conclusive results on attention: As mentioned, previous research has connected head angles of rodents to attention (section 4.8). However, given the fact that rats can rotate their eyes independently and have a maximum visual field as wide as 176 degrees (Hughes 1979, Wallace *et al.,* 2013), it is not farfetched to assume that an observer could have paid attention with their backs turned against the performer. However, the current analysis considered the animals' attentiveness as one of the factors, rather than the only factor that influences learning. A curious point to note is that if attention was the same across all observers in the observational group, how is it that two animals were still non-learners (2.7% and 7.7%; Supplementary Table C1, Appendix C)? Personality differences between animals could be a possible explanation, however, additional analyses are required to tackle this question.

4.11 Future studies

For the current project, the proposed manual analysis method is a worthwhile tool to study attention in this observational paradigm. Conclusive results about the role of ACC in observational learning are still being investigated in the PhD project that this thesis' work is built upon. With this tool, attentiveness of the observer can be quantified across groups, and build up on the results presented in this thesis. The manual analysis method saves time and reduces bias to a good degree, making attention analysis more robust.

Apart from this, the analysis method used to quantify attention in this thesis could prove to be an effective tool to study other cognitive behaviors. The results shown here clearly demonstrate that the analytical method is an effective tool to study rodent behavior. It combines computational tools to create specific rules for human annotations, reducing experimenter bias. The author proposes that the manual analysis method is most effective for shorter videos and can be used to study attention with specific detail. For example, by using light-ON and head direction with respect to stimulus balls (LO_SB; 2.11.1) processed videos, it was possible to check if the observer was paying attention to one of the balls. Thus, in a case where the performer was removed, this tool would be effective for measuring visual attention to objects or other stimuli in the environment. Other details of rodent-rodent interactions could also be explained using this tool, for example, whether interactions between performers and observers increases the subsequent attentiveness of the observer. On that front, use of this software on classical and operant conditioning tasks are also possible (depending on the behavioral apparatus).

The RAQ software could also be developed to better account for uncalculated angles and performer movements. It would be interesting to see how close the manual and automated attention scores could become if the accuracy of the software was improved. As mentioned, the angle of the recording camera could also potentially improve the accuracy of the RAQ software. This could be tested, for example, by using cameras with different viewpoints (like over-head or a combination of both over-head and bird-view). Nevertheless, the author believes that the RAQ software still shows great potential as a behavioral analysis tool, especially when combined with human annotators.

Studies have demonstrated that ACC is important for observational learning in fear-based paradigm (Jeon *et al.,* 2010, Allsop *et al.,* 2018, Keum *et al.,* 2018). In addition, Schneider and colleagues have speculated that ACC contributes to both reward and punishment in an observational experiment (Schneider *et al.,* 2020). Our preliminary results expand on these findings by suggesting an active role of ACC in reward-based observational learning. An interesting point is that irrespective of the presence of relevant

social cue, rats do maintain a certain degree of attention towards the performer, which is not affected due to ACC inhibition. Our results suggest ACC inhibition disrupts observational learning in a way that is not explained by attention (measured as head direction of the observer).

Previous studies have implied that increased activity in certain subgroups of neurons in ACC was associated to social attention (Schneider *et al.,* 2020). This interpretation was made because attention was a constant variable between stimulus cues of both rewarding or punishing nature. That is, subgroups of neurons that fired similarly for both cues were considered relevant for social attention. Results from this thesis supports the idea that attention is consistent across groups and builds up on the potential role of ACC in processing social attention. ACC inhibition does seem to disrupt observational learning, but the general attention of the animal was still intact. Multiple studies have linked ACC to target detection (Posner and Petersen 1990, Schweimer & Hauber 2005, van Heukelum *et al.,* 2020). The author speculates that task-relevant social information was lost or not processed by the observer due to the lack of fully functioning ACC. It could be possible that the observer was paying attention to the performer but could not associate the actions of the performer for receiving the reward (that is pushing the stimulus balls) to the target (stimulus balls). That is, in our study, ACC inhibition could have hindered the ability of the animal to accurately process the relationship between task-relevant social cues to the actual task of pushing the stimulus balls. Connecting this interpretation to the study by Schneider and colleagues (2020), it could be possible that there are subgroups of ACC that specifically helps correlate the task-relevant social information to the actual task. Since ACC have been shown to play an active role in a multitude of cognitive functions (Burgos-Robles *et al.,* 2019, van Heukelum *et al.,* 2020), this interpretation could be plausible. As a subsequent study, it would be interesting to see if there are any differences in the neural activity of ACC when a performer pushes the lit stimulus ball and when the stimulus ball lights up on its own. If there are subgroups of neurons in ACC that fire explicitly for target (stimulus balls) and social cues (the performer), future studies could focus on whether ACC acts like a 'junction' where the relationship between task and observed action of the performer needs to be processed. Conclusive results on the role of ACC for the paradigm explained in this thesis could prove critical for answering these questions.

Studies have also shown that intracranial stimulation of medial prefrontal cortex (mPFC) in rodents also disrupts observational learning (Jurado-Parras *et al.,* 2012, Yamada & Sakurai 2021). The correlation between this result and ACC inhibition would be an interesting front to explore. While these studies did not emphasize on the role of ACC in observational learning, Yamada and Sakurai recommended ACC to be a potential candidate for social information processing. The author agrees that studying the nature of projections from left prelimbic area of the mPFC to ACC, could shed some light on social information processing. That is, does mPFC stimulation modulate the activity of ACC in some way? If so, is that the reason for disruption of observational learning? Continuation of this project could bring into light some of the answers to these curious questions.

Section 5

Conclusion

Understanding the neural processing underlying observational learning is important for several reasons, the most critical being the fact that this form of behavior is required for survival in many species. To do this, it is first necessary understand these processes at a behavioral level. The primary aim of this thesis was to isolate and quantify behavioral factors that could affect learning in an established observational learning paradigm. Specifically speaking, we investigated the importance of social information and visual attention. The results indicate that social information, in the form of a trained demonstrator performing the task, is the most likely the reason for observational learning, since attention did not vary across groups. Automated software tools, such as the program we used here, could also be effective tools for behavioral analysis, but only after they have been carefully checked and compared against manual scoring. Depending on time and resources, manual scoring analyses, such as the method we used here, were also effective, though steps must be included to remove obvious biases in user labeling. In our case, this was done by the combining unbiased tracking data (RAQ software calculations) and precise manual scoring of behavior.

The secondary aim of the thesis was to examine the effect of ACC inhibition on attention, using data from the first five ACC-inhibited observers in the project. We fulfilled this aim using the manual analysis approach developed during the primary objective. The results suggest that ACC inhibition disrupts observational learning, since the performance of the performers was similar for both observational and ACC-inhibited groups. Furthermore, ACC inhibition did not appear to cause deficits in social attention of the animals. Correlations between the overall attention of the observers and their eventual performance were non-significant, but additional data are required to draw statistically sound conclusions. Answers to these and other questions should become clearer as the remaining experiments in the project are completed by Ida V. Rautio in the months to come.

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APPENDIX A

Supplementary protocols *see Appendix B for manufacturer details*

Supplementary protocol 1: Surgery

*General preparation of room one day before surgery included autoclaving of tools in a toolbox (at 120-135 degrees); filling isoflurane into vaporizer; filling a bottle 500mL 70% ethanol; preparing the surgery table with scalpel blade, cotton swabs, sugi, sponge dental, syringe and needles (3x1mL for injection of medication, 1x5mL for H_2O_2, 2x10mL for sterile H_2O, and respective needle tips), an extra needle tip, surgical blade, chlorhexidine, and finally placing a cotton cloth on heating pad. The room was sterilized with UV light everyday between 01:00am to 03:00 am.

**General preparation of room on the day of surgery included weighing the animal; turning heating pad, anesthetic gas vaporizer, oxygen concentrator, and microscope camera on; sterilizing the cotton swabs with UV-light and placing it back into room before surgery; preparing Marcain (1-3mg/kg), Temgesic (0.01-0.05mg/kg), and Metacam (1mg/kg); preparing syringe with H_2O_2 and sterile H_2O ; preparing an ice bucket to chill sterile water filled syringe; preparing table with Kwik-Sil and autoclaved tools (on surgical drape).

***General protocol for anesthesia included setting the flow rate of anesthetic gas vaporizer to 0.5-0.6 L/min (for surgeries flow rate oxygen concentrator is also initially set at 0.5-0.6 L/min along with anesthetic gas); once the attached chamber was saturated with isoflurane (generally 5-10 minutes), the animal was placed in the chambers until unresponsive.

****General cleaning of both surgery and perfusion room included cleaning and drying of tools; switching off all appliances including vaporizer, heating pad, pumps, and oxygen concentrator; filling isoflurane into vaporizer; cleaning the surgery table with 70% ethanol; restocking of used materials; disposal of used covers ice and used tissues; and cleaning chambers used for anesthesia.

*****Post-operative care included the animals being given Temgesic (0.3 mg/ml, subcutaneously) 6-12 hours after surgery and after 24 hours, Metacam (2 mg/mL, subcutaneously) was given. Immediately after surgery the animals were placed in a heating chamber at 30-35 degrees till anesthesia wore off. In case of post-operative infections Baytril (25 mg/kg) was provided along with intermittent injections of saline till recovery. Food intake was maintained using porridge, and in case of serious complications (like seizures or heavy bleeding that did not stop), perfusion was carried out.

1.a: Intracranial injection of Optogenetic viral vector

Animals: 9-week-old male Long Evans rat

Viral vector: AAV5-mDIx-Chr2-mCherry-Fishell-3, with an mDlx enhancer that targets GABAergic interneurons, prepared at Viral Vector Core Facility, by Rajeevkumar R. NairKavli Institute for Systems Neuroscience)

Procedure:

1. Before surgery the viral vector was prepared by mixing a very small amount of fast blue to the defrosted viral vector alliquote, using a centrifuge. Using a pipette, 700nL of the freshly prepared aliquot was removed and carefully inserted into a glass capillary micropipette. The capillary micropipette was then carefully filled with mineral oil to avoid backflow. Two such micropipettes were prepared (one for each hemisphere)
2. The surgery the room was prepared as described ** and the animal was anesthetized***
3. Once the animal was unresponsive, it was removed from the chamber, the head was shaved and placed on the heating pad with its teeth placed in the mouthpiece of the stereotaxic
4. The head was fixed on the stereotaxic frame using ear bars to get a level skull, and the heating pad was readjusted to allow proper airflow and breathing, and gas flow was adjusted to lower flow rates of 03-04 L/min for both oxygen and isoflurane.
5. Viscotear was applied to eyes, the area was trimmed for remaining hair, and claws were cut
6. Metacam and Temgesic was provided subcutaneously, while Marcain was injected at incision site as a local anesthetic. The injection site was cleaned with chlorhexidine after Marcain had diffused into tissue and the animal was ready for surgery.
7. Incision site was opened with a surgical blade and scalpel, cleaning off blood continuously
8. Area was further exposed by attaching four clamps to corners and taping them to the frame
9. H2O2 was applied and used to disinfect the skull, followed by saline to clean it
10. Step 10 was repeated till most dead tissue was removed using curved tip microscissors
11. The bregma was marked using a drill attached with a 1mm burr by making a small dent in the skull surface.
12. The drill was moved to injection site using the marked bregma as reference point (AP: +2.5)
13. Two holes were made at ML: +0.5 and -0.5
14. The craniotomies were cleaned for excess bone residue and tissue
15. A needle was used to slightly cut the dura.
16. The microinjection syringe was assembled with prepared capillary micropipette (with the viral solution inside)
17. Syringe was attached to stereotaxic frame that was connected to microinjection pump
18. Micropipette was moved to the site of injection using bregma (AP: +2.5, ML: +/- 0.5)

19. The capillary micropipette was then lowered slowly into the brain (DV: +2) and the 700nL aliquot was injected into the target area at 50nL/min flow rate.
20. After injection, the micropipette was slowly retracted after 10 minutes to avoid backflow
21. Step 15 to 20 was repeated with the second capillary micropipette for the other hemisphere
22. The craniotomies were covered using Kwik-Sil and the incision was sutured.
23. The area was rinsed with saline till clean, disinfectant cream was applied, ear bars removed, and the animal was moved to heating chamber till recovery from anesthesia
24. Finally, general cleaning of the room was done**** and post-operative care was given*****

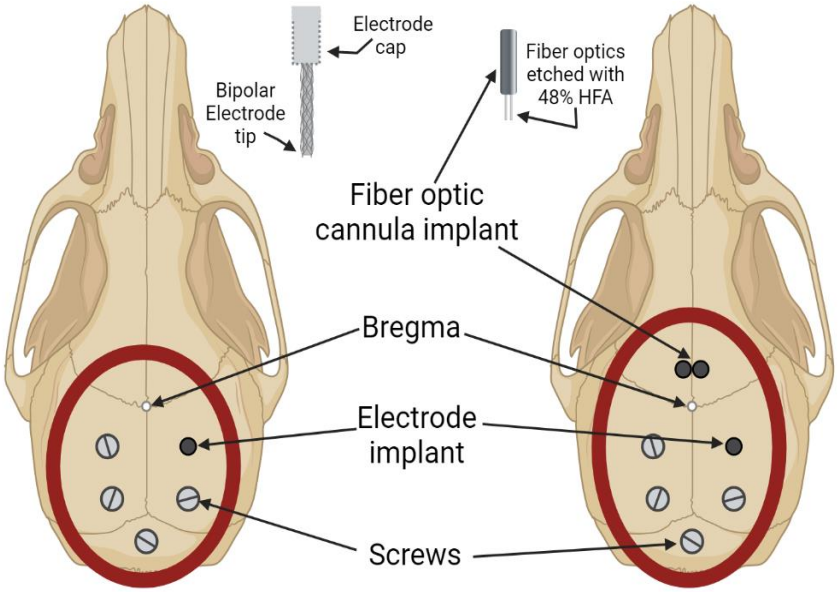
1.b: Intracranial implantation of bipolar electrode

Animals: Male Long Evans rat, minimum 12-week-old, ≥400 gms in weight

Electrode: Stainless-steel bipolar stimulating electrode

Procedure:

1. One day prior to surgery an electrode was selected, and the room was prepared accordingly*
2. Next day, the electrode was attached to a stereotaxic arm, and submerged into 70% ethanol until use. The room was prepared accordingly** and animal was anesthetized***
3. Steps 3 to 11 from supplementary protocol 1.a. was carried out.
4. The drill was moved to coordinates AP: -2.8, ML: +1.7, and a craniotomy was made such that dura was exposed with minimal bleeding.
5. Additional holes were drilled as illustrated in supplementary Figure A.1. The area was flushed with cool saline during drilling to avoid burned bone.
6. The holes around the main craniotomy were attached with screws using a surgical screwdriver, turning 2.5-3 turns to secure them to the skull.
7. The area was cleaned once more and the prepared stereotaxic arm with electrode from step 2 was attached to the stereotaxic frame
8. Electrode was moved to coordinates AP: -2.8, ML: +1.7 using bregma as reference point.



Supplementary Figure A.1: Illustrates the implantation sites for electrode (left) and fiber optic cannula (right). The *black circles* represent the approximate location of the craniotomy made for the respective implants, while the *silver circles* represent the approximate locations of screws intended for anchoring the implants to the skull. The *white circle* represents the bregma (or coordinate AP: 0, ML: 0, DV: 0).

9. The dura was slightly punctured, and the electrode was slowly inserted into the brain to a target coordinate of DV: + 8
10. Excess blood was cleaned carefully, and the electrode and craniotomy were coveredwith Kwik-Sil
11. The base of the electrode cap, dried Kwik-Sil surface, screws and skull area near the implant was covered with a thin layer of prepared Super-Bond to strengthen the binding of dental cement to the screws and skull. After drying, the implant was covered in self curing dental cement.
12. After the cement had dried, the electrode was slowly removed from the stereotaxic arm
13. Using cotton swabs, the cement was checked for sharp edges which were removed using drill and saline, and the area was cleaned using saline
14. The curved-tip clamps along with any dead tissues were removed and area was cleaned
15. The incision was sutured if needed and cleaned one last time before removing ear bars, and moving the animal to the heating chambers
16. Finally, general cleaning of the room was done**** and post-operative care was given*****

1.c: Intracranial implantation of bipolar electrode and fiber optic cannula

Animals: 12-week-old (minimum age) male Long Evans rat injected with viral vector (1.a), ≥400 gms in weight

Electrode: Stainless-steel bipolar stimulating electrode

Fiber optic cannula: Fibers were etched with 48% hydrofluoric acid

Procedure:

1. One day prior to surgery a selected electrode, and previously selected optic fiber (section 2.3.1) were placed in the room and the room was prepared accordingly*

2. Next day, the electrode and cannula were attached to two separate stereotaxic arms and submerged into 70% ethanol until use. The room was prepared accordingly** and animal was anesthetized***
3. Steps 3 to 14 from supplementary protocol 1.a. was carried out.
4. Steps 4 to 6 from supplementary protocol 1.b. was carried to complete the craniotomy for both fibers and electrode (see supplementary Figure A.1, right illustration)
5. The area was cleaned, prepared stereotaxic arm with cannula was attached to stereotaxic frame, and stereotaxic arm was adjusted to an angle of 20 degrees along the anterior-posterior-axis (entry angle of fibers)
6. The fibers were moved to coordinate AP: +3.2-3.3, ML: 0
7. The fibers were then slowly inserted into the brain to target coordinate DV: + 2-2.1, at a 20-degree angle
8. Once inserted, steps 7 to 10 of 1.b. was done to carefully complete electrode implantation
9. The excess blood was cleaned carefully, and the electrode and fibers were covered with Kwik-Sil
10. The base of the electrode cap and cannula, dried Kwik-Sil surfaces, screws, and skull area near the implants were covered with a thin layer of prepared Super-Bond to strengthen the binding of the cement to the screws, implants, and skull. After drying, the area near the implants were covered in self curing dental cement.
11. After drying, cannula followed by the electrode were released from the stereotaxic arm
12. Steps 13 to 14 of 1.b. was carried out followed by general clearing of the room**** and post-operative care was given*****

1.d: Solutions for Perfusion

The animals were perfused immediately after the experimental procedures were completed (section 2.10.1). Solutions used were:

500mL Ringer solution for substituting blood and cleaning vessels: 4.25gm NaCl (0.85%), 0.125gm KCl (0.025%), 0.1g $NaHCO_3$ (0.02%) is mixed with 500mL distilled water. The solution is filtered, heated to 40 degrees, and pH was set to 6.9 using oxygen flow immediately before perfusion.

200mL 10% Paraformaldehyde (PFA): Add 20gm PFA to 200mL distilled water along with 2-4 drops of NaOH. Stir till the solution is clear.

200mL 4% Paraformaldehyde to fixate the brain: Mix the 200mL 10% PFA to 156mL 0.4M phosphate buffer and 146mL distilled water. Set pH to 7.4 using a pH meter with HCl and NaOH. Filter before use.

Supplementary protocol 2: NISSL staining

1. Ten glass containers were prepared, two filled with Xylene; two with absolute alcohol (1000% ethanol); 70% ethanol with acetic acid; one of each: 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol; and finally crystal violet stain (0.5M crystal violet acetate, 200mL, placed on a shaker)
2. The prepared slides were dehydrated (placed in slide holder) by dipping the slides 10 times into; 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol, 100% ethanol, and finally 100% ethanol
3. Slides were shifted to xylene for 2 minutes for cleaning followed by rehydration step (reverse steps in dehydration, i.e., 100%→ 100%→ 90%→ 80%→ 70%→ 50% ethanol)
4. Slides were washed in running water and placed inside crystal violet, in dark, for 3min
5. Excess staining was removed in running water and slides were moved to an ethanol and acetic acid solution for a few seconds. If good color and contrast was not achieved, steps 4 and 5 were repeated till desired results were achieved
6. The slides were again dehydrated as in step 2 and clean the sections in Xylene for 2 min
7. The sections were then moved to a second Xylene bath for 5min to 1 hour
8. Finally, the slides were removed from Xylene and cover slipped using Entellan
9. Once dried (overnight), excess Entellan was scraped off and the slides were cleaned using ethanol before scanning in an automated slide scanner (Zeiss Axioscan Z1, Germany).

Supplementary protocol 3: GFP-RFP-Immunostaining

Introduction: Primary antibody binds to the protein where green fluorescent protein (GFP) is present. Secondary antibodies bind to the secondary antibody and amplify the fluorescence.

Method (notes by Merethe Andresen):

Day 1 - RFP primary antibody (Dilution at 1:1000, stored at +4°C)

1. Perform day1 protocol 20- to preferably 24hours before day 2 protocol.
    a. Take solution 1 out of the freezer about 1h before you start: 20ml for 1 brain.
    b. Wash 3 x 5 min in PBS in well (on shaker, 100 rpm, RT)
    c. Just enough shaking to see gentle movement in slices.
    d. Wash 2 x 10 min in solution 1 (on shaker, 100 rpm, RT)
    e. Incubate with primary antibodies (on shaker, 60 rpm, at 4°C) overnight
        i. Prepare solution with AB max 10 minutes before use in glass with lid. Label the glass. The glass gives more movement of slices than the well.
        ii. Tape glass to shaker. Label it.

Day 2: Goat Anti-rat IgG secondary antibody (Dilution 1:1000, stored at +4°C, AlexaFluor, 546 nm)

1. Take solution1 and 2 out of the freezer about 1h before you start: 20ml is enough

for 1 brain.

2. Wash 2 x 5 min in solution 1 (on shaker, 100 rpm, RT) To remove old solutions.
3. Incubate with secondary antibodies (on shaker,60 rpm, RT) for 1 hr in solution 2
    a. Tape glass to shaker. Cover glass with an aluminium foiled box.
4. Wash 2 x 10 min in PBS (on shaker, 100 rpm, RT)
5. Mount on gelatin-coated polysine slides using PBS
    a. Solution: 0,5gr gelatin+50ml distilled water. Dissolve on a stirrer at 60°C. Apply with a clean brush and dry at 100°C.
6. Dry overnight
7. Apply Hoechst (in the dark in the hood)
    a. Put the slides in the hood and cover with Hoechst solution
    b. Wait 5 min, rinse gentle with PBS

Solutions

Solution 1 : PBS 0.1 M + 0.3% Triton. For 100 mL of solution 1: 97 mL PBS + 3 mL of 10% Triton.

Has detergent (Triton) to remove lipids and ease AB binding.

Solution 2 : PBS 0.1M + 0.3% Triton + 3% BSA. For 50 mL of solution 2: 32.5 mL PBS + 1.5 mL 10% Triton + 15 mL 3% BSA.

BSA: 3% BSA: mix 1.5g BSA in 50 mL PBS

BSA can be from any species, except from rodents. Blocking agent – makes AB binding more specific by binding to all unspecific places.

Supplementary protocol 4: GitHub location for Rodent Attention Quantifier codes

<https://github.com/mokkalokka/RodentAttentionQuantifier.git> (includes 50 files with codes)

Maker: Michael S Larsen, mokkalokka (GitHub profile name)

Supplementary protocol 5: MATLAB code for head angle plots, attentiveness calculation, scatter plot, box and whisker plot, and NaN angle calculations

```
%Head angle data
Data_tab = readtable("OptoExp7-3_angles.csv");
Data = table2array (Data_tab);
Angles = Data(:,2);
figure;
h = histogram(Angles, 180, 'FaceAlpha', 0.3,'EdgeAlpha',0.4,'FaceColor',
'k');
title ('Head direction distribution');
xlabel ('Head angle (deg)');
ylabel ('Frequency');

% calculate percentage of attentiveness categories from the software
a_threshold = 40;
pa_threshold = 110;
pu_threshold = 140;
```

```
totalCounts = sum(h.BinCounts);

percent_attentive = sum(h.BinCounts(h.BinEdges > 0 & h.BinEdges <=
a_threshold)) / totalCounts;
percent_possattentive = sum(h.BinCounts(h.BinEdges > a_threshold + 1 &
h.BinEdges <= pa_threshold)) / totalCounts;
percent_possunattentive = sum(h.BinCounts(h.BinEdges > pa_threshold + 1 &
h.BinEdges <= pu_threshold)) / totalCounts;
percent_unattentive = sum(h.BinCounts(h.BinEdges(1:60) >= pu_threshold))
/ totalCounts;

% Color Coded histogram
figure;
histogram(Angles(Angles <= a_threshold), a_threshold, 'FaceAlpha', 0.6,
'EdgeAlpha',0.4)
hold on;
histogram(Angles(Angles > a_threshold & Angles <= pa_threshold),
pa_threshold - (a_threshold + 1),'FaceColor', 'g', 'FaceAlpha', 0.4,
'EdgeAlpha',0.4)
hold on;
histogram(Angles(Angles > pa_threshold & Angles <= pu_threshold),
pu_threshold - (pa_threshold + 1),'FaceColor', 'y', 'FaceAlpha', 0.4,
'EdgeAlpha',0.4)
hold on;
histogram(Angles(Angles > pu_threshold), max(h.BinEdges) -
pu_threshold,'FaceColor', 'm','FaceAlpha', 0.4, 'EdgeAlpha',0.4)

title('Head direction distribution');
ylabel('Frequency');
xlabel('Head Angle (degrees)');
```

```
%TIME SPENT ON TASK SIMPLE
T_spent = readtable("Avg time on task-Time spent on task.csv");
T_spent_tab = removevars(T_spent,"Var1");
T_spent_Data = table2array (T_spent_tab);
T_spent_Data_Tras = transpose(T_spent_Data);
figure;
plot(T_spent_Data,'o-k', "Marker", "o", "Color",
"black","MarkerFaceColor", "red",'LineStyle', 'none');
title('Average trial length');
xlabel('Groups');
ylabel('Time (sec)');
xlim([0 4]);
ylim([0 80]);
set(gca,'FontSize',20)
hold on;
boxplot(T_spent_Data_Tras);
```

```
% Calculate number of NaN angles
Data_tab = readtable("OptoExp7-3_angles.csv");
Data = table2array (Data_tab); % convert to angles?
Angles = Data(:,2);
No.NaN=sum(isnan(Angles));
Per.NaN=(sum(isnan(Angles))/numel(Angles))*100;
```

```
%Performance (obs vs per) with attention
```

```
PerformanceObs = [100; 96.67; 98.5; 95.73; 95.63; 78.83];
ObserveperfObs = [60; 47.5; 7.7; 41.7; 2.7; 25.6];
PerformanceACC = [93.3; 99.2; 99.6; 100; 95.77];
ObserveperfACC = [0; 0; 89.8; 2.6; 0];
Attentionobs = [65.38; 69.49; 66.99; 70.04; 61.23; 70.74];
AttentionACC = [72.46; 62.67; 66.88; 63.54; 63.58];
figure;
scatter (PerformanceObs, ObserveperfObs, 200, Attentionobs, 'filled');
%200 is the marker size
xlim([75 100]);
ylim([0 100]);
colorbar; 4
lim = caxis,
caxis([0 100]);
hold on
scatter (PerformanceACC, ObserveperfACC, 200, AttentionACC,'d','filled');
colormap autumn;
oldcmap1 = colormap;
colormap(flipud(oldcmap1));
```

APPENDIX B

Supplementary materials

Animals

Supplementary Table B1: List of observers used for experiments

Identification number (ID), Date of Birth (DOB), Date of Viral Injection (DOVI), Date of Implantation (DOI), Date of Death (DOD), Electrode strength (ES) used for experiments

Animal ID	DOB	DOVI	DOI	Surgeon	ES (mA)	DOD
Observational observers						
26737	15-06-2020	X	13-09-2020	Devika K	24	02-11-2020
26770	03-06-2020	X	01-09-2020	Ida V. Rautio	26	19-10-2020
27061	12-10-2020	X	04-01-2021	Ida V. Rautio	26	08-02-2021
27063	12-10-2020	X	06-01-2021	Ida V. Rautio	28	03-02-2021
27171	27-11-2020	X	24-02-2021	Ida V. Rautio	24	15-03-2021
27219	23-12-2020	X	18-03-2021	Ida V. Rautio	52	04-06-2021
Control observers						
26669	20-05-2020	X	17-08-2020	Devika K	22	19-10-2020
26672	20-05-2020	X	20-05-2020	Ella H. Holmberg	24	19-10-2020
26612	29-04-2020	X	29-07-2020	Ella H. Holmberg	22	04-09-2020
26771	03-06-2020	X	27-08-2020	Ella H. Holmberg	24	28-09-2020
27286	07-03-2021	X	01-06-2021	Ida V. Rautio	26	01-08-2021
27336	29-03-2021	X	08-07-2021	Ida V. Rautio	24	26-07-2021
ACC inhibited observers						
27174	27-11-2020	31-01-2021	07-03-2021	Ida V. Rautio	26	22-03-2021
27216	23-12-2020	01-03-2021	08-04-2021	Ida V. Rautio	30	20-04-2021
27260	28-01-2021	04-04-2021	10-05-2021	Ida V. Rautio	26	26-05-2021
27323	21-03-2021	28-05-2021	28-06-2021	Ida V. Rautio	18	12-07-2021
27322	21-03-2021	27-05-2021	02-07-2021	Ida V. Rautio	25	15-07-2021

Supplementary Table B2: List of performers used for experiments

Identity number (ID); Date of Birth (DOB); Date of Implantation (DOI); Date of Death (DOD), Electrode strength (ES); Observational experiment (Ob); Control experiment (C); ACC experiment (A)

Animal ID	DOB	DOI	Surgeon	ES (mA)	DOD	Times used
26668	20-05-2020	10-08-2020	Devika K	24-26	02-11-2020	2 Ob,1 C
26994	18-09-2020	15-12-2020	Ida V. Rautio	26	25-01-2021	1 Ob
27062	12-10-2020	08-01-2021	Ida V. Rautio	26	23-02-2021	1 Ob
27075	13-10-2020	17-02-2021	Devika K	26-28	08-04-2021	2 Ob, 1 A
26671	20-05-2020	11-08-2020	Ella H. Holmberg	26	19-10-2020	1 C
26613	29-04-2020	30-07-2020	Ella H. Holmberg	24	19-08-2020	1 C
26721	01-06-2020	24-08-2020	Ella H. Holmberg	24	19-10-2020	1 C
27287	07-03-2021	21-06-2021	Ida V. Rautio	26	31-05-2021	1 C
27335	29-03-2021	26-07-2021	Ida V. Rautio	24	05-07-2021	1 C
27219*	23-12-2020	18-03-2021	Ida V. Rautio	42	04-06-2021	1 A
27217	23-12-2020	22-03-2021	Ida V. Rautio	24	12-07-2021	2 A
27286*	07-03-2021	01-08-2021	Ida V. Rautio	24-26	01-06-2021	1 A

**trained observers from other groups reused as performer*

Supplementary Table B3: List of unused/practice/operated animals with non-function electrodes or motor artefacts

Animals with working electrodes that were intended as performers but were unused due to lack of learning the task after multiple weeks of training or behavioral issues (like jumping out of the box, aggression, and progressively increasing dominance behavior). Identity number (ID); Date of Birth (DOB); Date of Viral Injection (DOVI); Date of Implantation (DOI); Date of Death (DOD); Electrode strength (ES); Surgeon: Devika Kurup

Animal ID	DOB	DOI	DOD	ES(mA)	Use/reason for death
26554	18-04-2020	08-11-2020	08-11-2020	—	Practice
26590	25-04-2020	19-11-2020	19-11-2020	—	Practice
26675	20-05-2020	15-10-2020	15-10-2020	—	Practice
27076	13-10-2020	25-01-2021	12-02-2021	24-26	Unused trainer
26885	20-08-2020	05-12-2020	05-01-2021	22	Unused trainer
26884	20-08-2020	28-11-2020	12-02-2021	24	Unused trainer
26883	20-08-2020	06-12-2020	05-01-2021	—	Motor artefact
26882	20-08-2020	13-01-2021	01-01-2021	26	Unused trainer
26803	17-07-2020	19-10-2020	23-10-2020	—	Motor artefact
27074	13-10-2020	19-12-2020*	12-02-2021	—	Unused

**Viral injection animal*

Resource Table

Equipment, Devices, and Reagents

Chemical/reagent, specification (manufacturer)

Saline 9 mg/mL, 100 & 10 mL (B Barun, Germany)

Ethanol 100% (KiiltoClean AS, Norway)

Sucrose ((VWR chemicals, USA)

Chlorhexidine digluconate, 5mg/mL (Fresenius Kabi, Germany)

Sodium hydroxideNaOH, 35% (VWR chemicals, USA)

Hydrogen peroxide, 3% (Apotekenes, Norway)

Pentobarbital sodium (Richter Pharma, Austria)

Triton X 100, ≥90% (Merck, Germany)

Xylene, ≥98.5% (VWR , USA)

Toluene, ≥99.5% (VWR , USA)

Sodium Chloride [NaCl], ≥99% (VWR , USA)

Sodium bicarbonate [NaHCO3], ≥ 99.7 % (Sigma, USA)

Potassium chloride [KCL], 99.5% (Sigma, USA)

Pyrisept, 1 mg/mL (Weifa, Norway)

Simplex (Ophtha, Norway)

Viscotears, 2 mg/g (Thea, Norway)

Virkon (Barge Medical AS, Norway)

Temgesic (Schering-Plough, USA)

Isoflurane 100% (Zoetis, USA)

Baytril, 25 mg/mL (Bayer, Germany)

Potassium hydroxide [KOH], ≥85% (Sigma Life Science, USA)

Paraformaldehyde [PFA], ≥95% (Sigma Aldrich, USA)

Dimethyl sulfoxide [DMSO], ≥99.7% (Sigma Aldrich, USA)

Entellan 100% (Merck KGaA, Germany)

Bovine serum albumin, ≥96% (Merck, Germany)

Goat Anti-rat IgG, Alexa Fluor 564 (ThermoFisher, USA)

Cresyl Violet 0.5g (Merck KGaA, Germany)

KH_2PO_4, ≥ 99.5 % (Merck KGaA, Germany)

Glycine, ≥99% (Sigma, USA)

Acetic acid, 100% (VWR, USA)

RFP antibody (Chromotek, Germany)

Goat anti rat IgG, Alexa fluor 546 (ThermoFisher, USA)

Fast green FCF (Sigma Aldrich, US)

Metacam, 0.5 & 2 mg/mL (Boehringer Ingelheim, Germany)

PBS, (Merck KGaA, Germany)
Phosphate buffer 0.4M ((Merck KGaA, Germany)

Red fluorescent protein [RFP] (Chromotek, Germany)

Equipment, specification (manufacturer)

Microtome (Thermo scientific, USA)

Scanner (Zeiss Axioscan Z1, Germany)

Flowmeters (MPB industries, UK)

Perfusion pump (World precision Inc, USA)

Downflow table (AFOS, UK)

Fume cupboard (Byrn Byggklima, Norway)

Freezer, EBL 18210 F (Hotpoint, USA)

Weight balance, gm (OHAUS CS 2000, USA)

Weight balance, mg (Sartorius, Germany)

Heat pad (VWR, USA)

pH meter (InoLab, Germany)

Micropipette puller (Sutter Instrument, USA)

Autoclave (TOMY, Japan)

Hardboard (TB4, Thor lab, Germany)

Stimulator, ISO-Flex (A.M.P.I., USA)

Lasers and DPSSL, Class 3b, BL473T3 - 150 (Sloc lasers, China)

Drivers, 170mw/131mw(Sloc lasers, China)

Fiber optic cannula, 200µm, NA = 0.3 (Doric lenses, Canada)

Fiber optic chord (Doric lenses, Canada)

Arduino/Genuino (Arduino, Italy)

Photodiode power sensor S142C (Thor Lab, Germany)

Energy Meter Console, PM100D (Thor Lab, Germany)

Infrared lights

Slides (Thermo scientific, USA)

Tape (3M, USA)

Grafting Tape (Parafilm, USA)

Device/tool, specification (manufacturer)

Camera, Raspberry Pi NoIR camera (Sony)

Raspberry Pi NoIR (Raspberry Pi Org, UK)

Pulse stimulator, master 9 (A.M.P.I., USA)

Surgery tool, specification (manufacturer)

Surgical blades and scalpels (Swann-Morton, UK)

Silicone adhesive, Kwik-sil (World Precision Inc, USA)

Dental acrylic cement (Kulzer GmbH, Germany)

Self-curing adhesive, Superbond C&B (Sun Medical, Japan)

Microinjector syringe and pump (World Precision Inc, USA)

Stereotaxic frame (David KOPF Inc, USA)

Anesthetic gas vaporizer (MSS Inc, UK)
Air duster (PRF, UK)

Oxygen concentrator (Pureline, Supera Anesthesia Inc, USA)

Slit lights (Carl Zeis, Germany)

Ventilated Cabinets (Scantainer Scanbur)

Microscope camera (Carl Zeiss, Germany)

Temperature controller (Physitemp Inc, US)

Drill (David KOPF Instruments, USA)

Burr, 1mm (Fine Science Tool, USA)

Bipolar stimulating Electrode (P1 Technologies, Canada)

Sterile Surgical Blades (Swann - Morton)

Injection needle (B Barun, Germany)
0.80x40mm 21 G1/2”
0.40x16mm 25 G5/8”
0.40x12mm 27 G1/2”

Injection needle, 1, 5 & 10mL (B Barun, Germany)

Injection needle, 2mL (BD Plastipak, Norway)

Sponge Dental (MS000, Ethicon, USA)

Sugi (KettenBach, USA)

Wipes (KimTech, USA)

Suture, 4/0 1.5 (Resorba, Germany)

Software, version

ZEN blue 2.3

BioRender 2020, https://app.biorender.com/

MatLab R2021a 9.10.0

IBM SPSS 27.0.1.0

DeepLabCut (2.2b8)

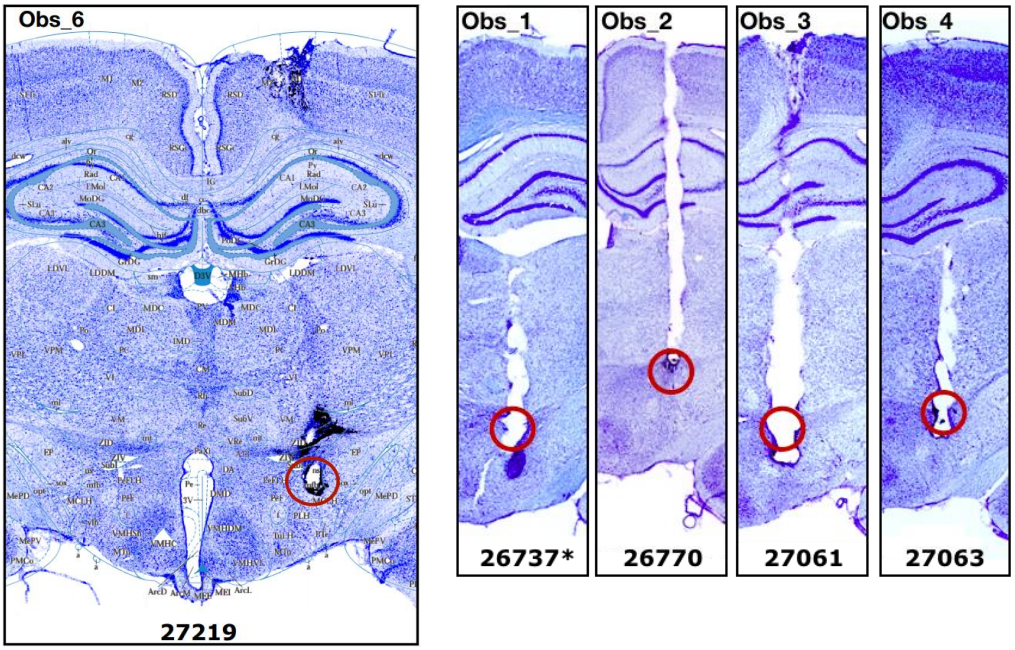
Tinkercad (online 3D modeling program), https://www.tinkercad.com/

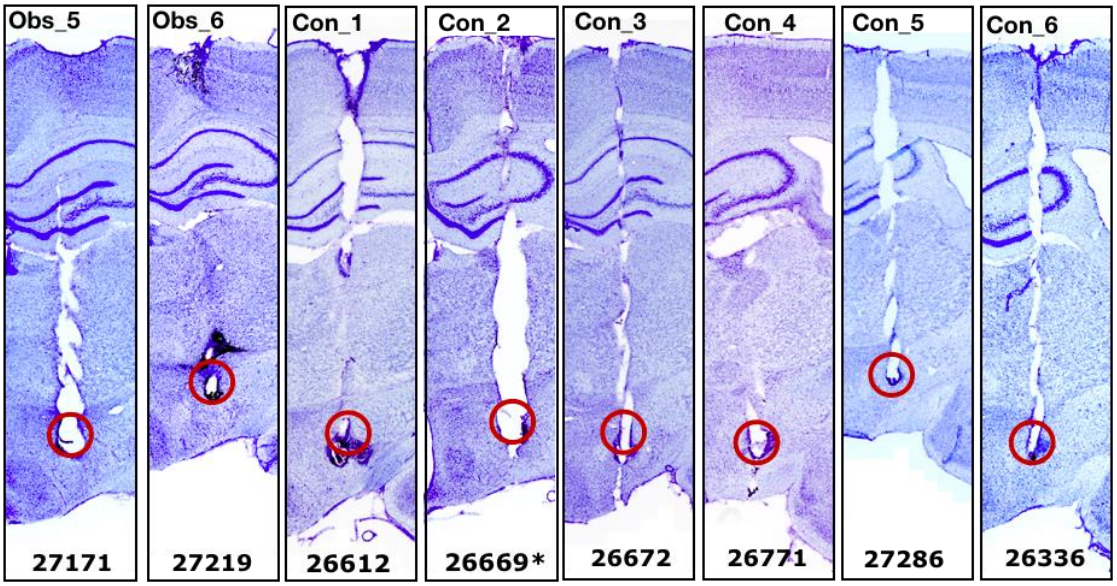
APPENDIX C

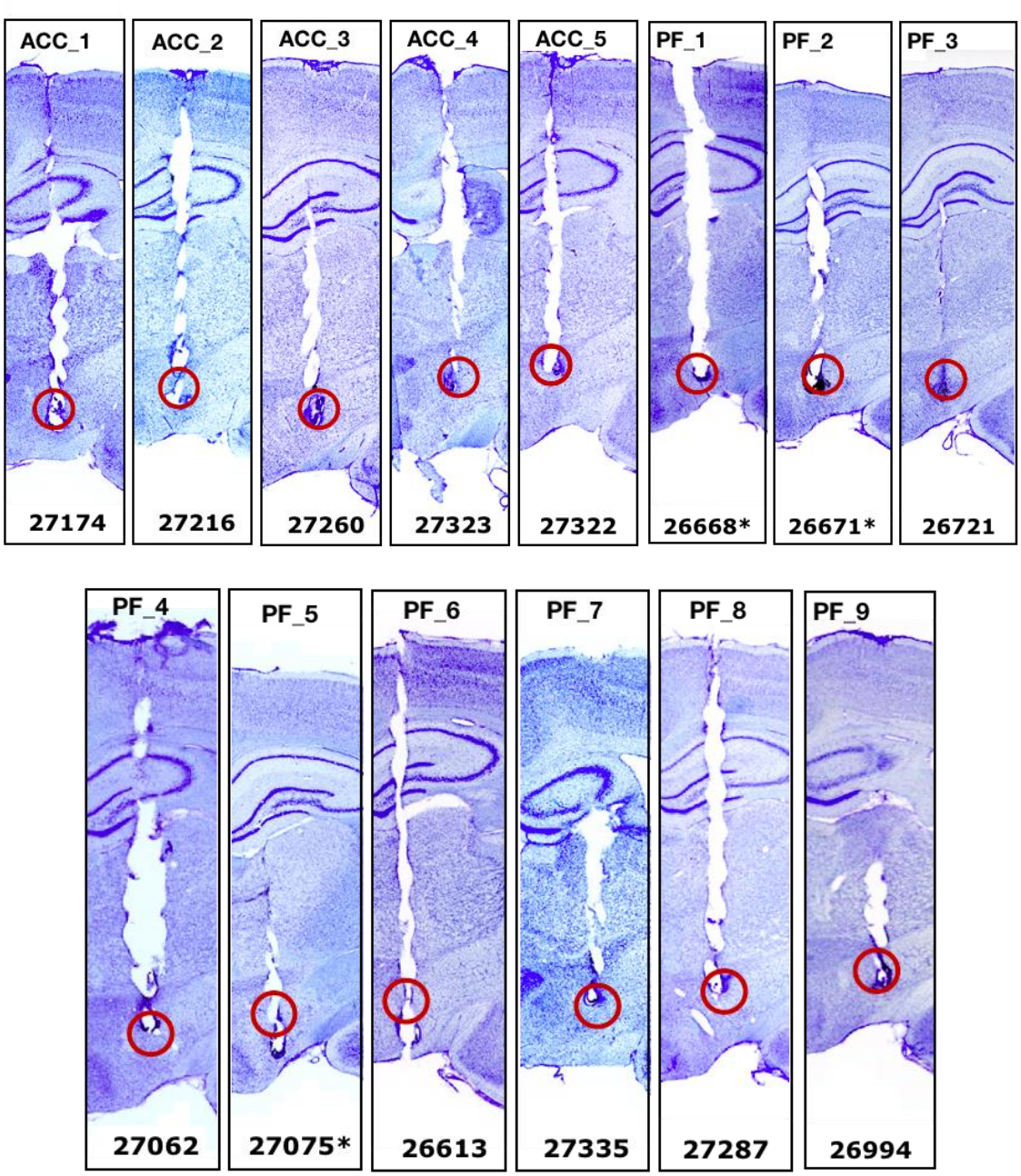
Supplementary Data

Supplemental data 1. Collection of histology scans

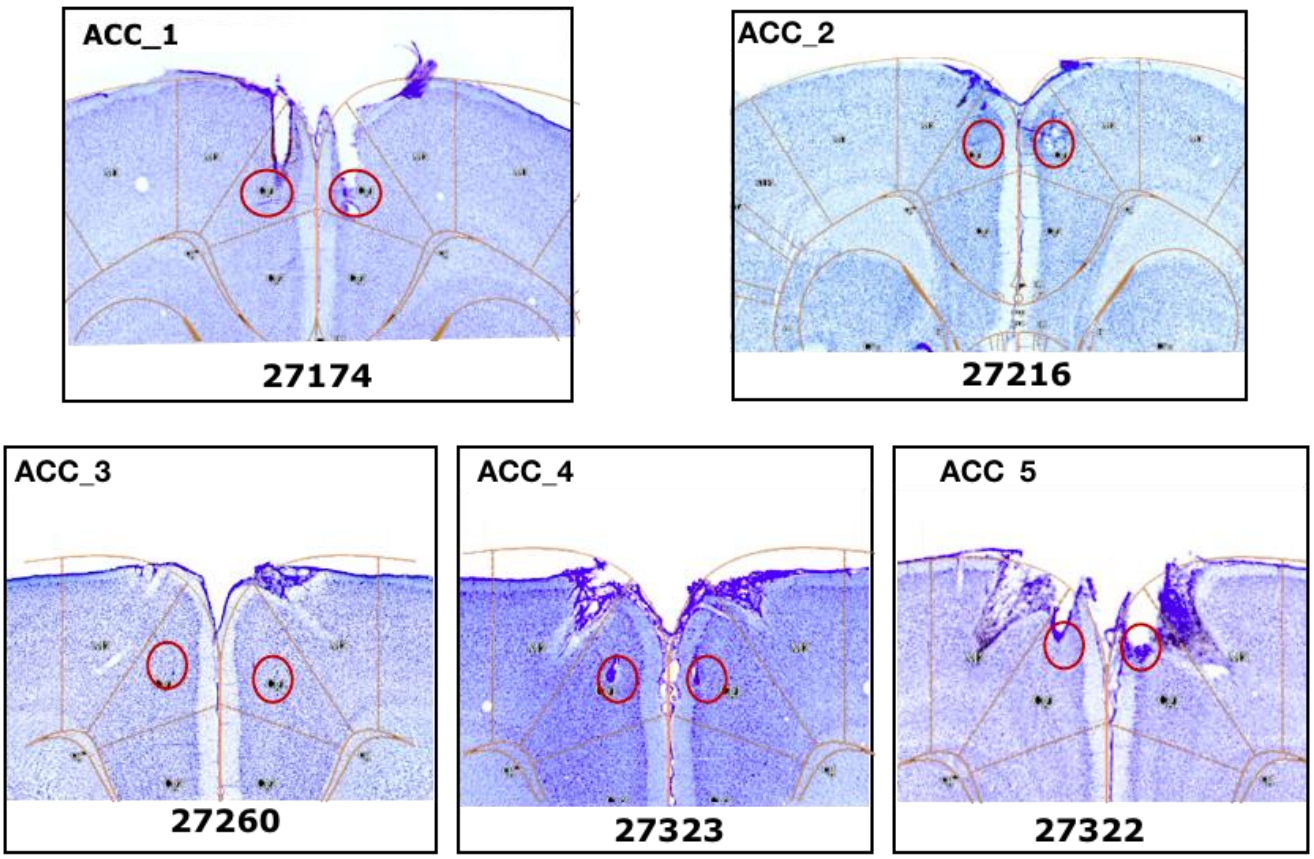
Supplementary Figure C1: Scans of NISSL-stained slices showing MFB implantation with bipolar stimulating electrodes (highlighted with red circle) for observers from observational group (Obs), control group (Con), ACC inhibited group (ACC); and performers (PF) with animal ID number at bottom. Ones marked with (*) were prepared by Devika Kurup, rest were prepared by Merethe Anderson. Unsliced brain: performer 27217.



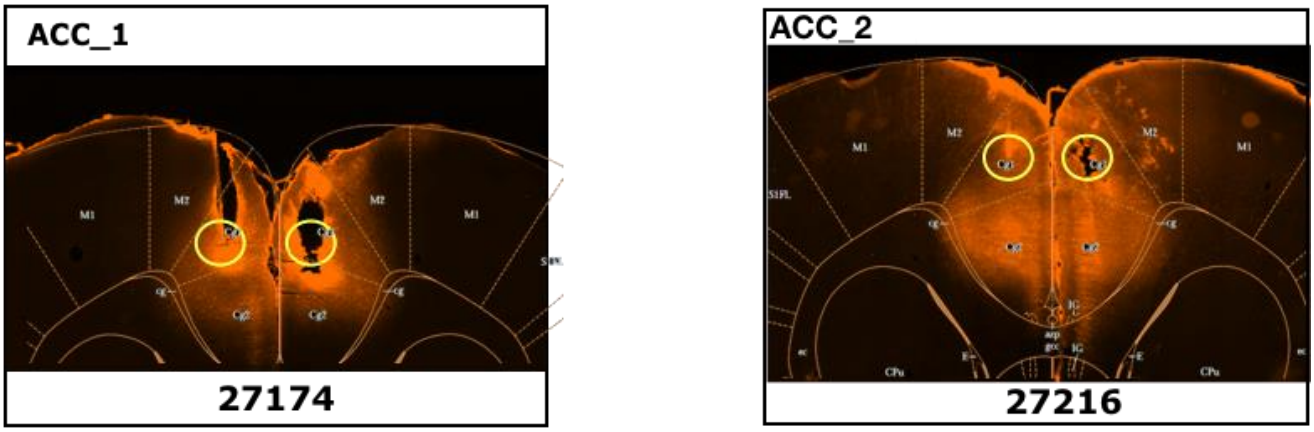


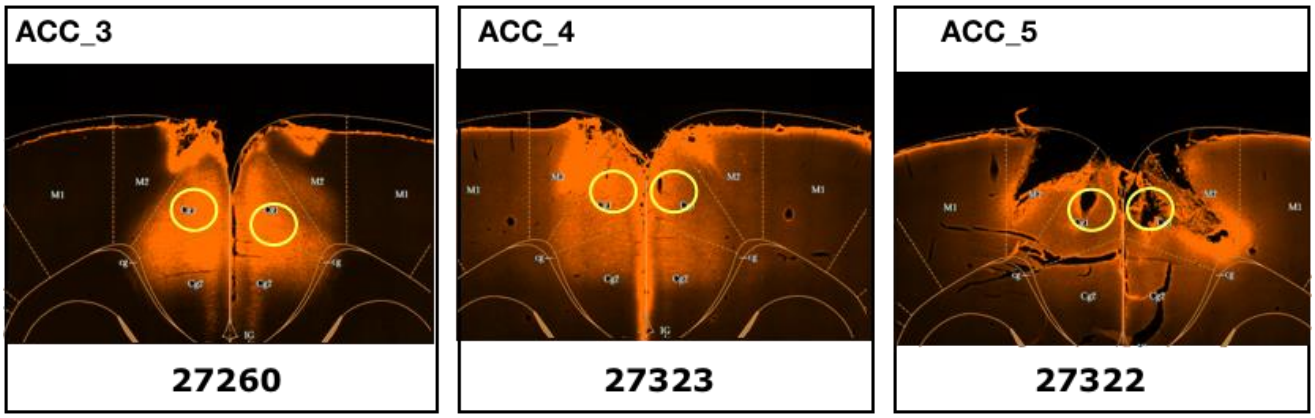


Supplementary Figure C2: Scans of NISSL-stained slices showing fiber-optic cannula implants at Cg1/Cg2 region of ACC (highlighted with red circle) for observers from ACC inhibited group (ACC) used in this thesis with animal ID number at bottom. Slides were prepared by Merethe Anderson.



Supplementary Figure C3: Immunostained slides of ACC inhibited observers with viral expression on Cg1/Cg2 of ACC region (highlighted with yellow circle) done with Immunohistochemistry with animal ID number at bottom and yellow circles highlighting the fiber implant. Slides immunostained and prepared by Merethe Anderson.





Supplemental data 2. Data from experimental groups

Supplementary Table C1: Data collected from experimental groups

Table shows the raw data collected during four days of experiment. Day 1 to 3 shows performance of the performer, and day 4 shows the testing day results of the observer. PST: Proportion of Successful Trials (performance, %); TS: Trial Speed (Stimulations/Session Length); ATL: Average Trial Length; SB2. L: Latency to tap Stimulus Ball 2 after first tapping Stimulus Ball 1.

			Day 1 to 3			Day 4 (testing day; 30 min)			
			PST% of performer			Observer data (Day 4)			
Experiment.	No.	Experiment performed by	Day 1	Day 2	Day 3	PST (%)	TS (St/SnL)	ATL (s)	SB2.L (s)
Observational experiment	1	Devika K	100.00	100.00	100.00	60.00	0.80	31.80	11.42
	2	Devika K	100.00	93.00	97.00	47.50	0.62	32.03	11.45
	3	Ida V. Rautio	97.60	97.90	100.00	7.70	0.10	48.31	20.70
	4	Ida V. Rautio	91.40	95.80	100.00	41.70	0.50	40.89	17.08
	5	Ida V. Rautio	100.00	86.90	100.00	2.70	0.03	51.25	23.37
	6	Ida V. Rautio	100.00	68.60	67.90	25.60	0.33	40.84	16.96
Control experiment	1	Devika K	100.00	100.00	100.00	0.00	0.00	58.13	30.07
	2	Ella H. Holmberg	100.00	100.00	100.00	0.00	0.00	59.19	30.09
	3	Ella H. Holmberg	100.00	100.00	100.00	0.00	0.00	60.00	30.00
	4	Ella H. Holmberg	100.00	100.00	100.00	13.20	0.16	48.26	21.61
	5	Ida V. Rautio	100.00	100.00	100.00	15.40	0.20	44.85	18.57
	6	Ida V. Rautio	100.00	100.00	100.00	0.00	0.00	59.49	30.07
Observational experiment with ACC inhibition	1	Ida V. Rautio	95.80	98.90	85.20	0.00	0.00	59.31	30.08
	2	Ida V. Rautio	100.00	97.60	100.00	0.00	0.00	59.45	30.09
	3	Ida V. Rautio	100.00	100.00	98.80	89.80	1.00	15.87	4.28
	4	Ida V. Rautio	100.00	100.00	100.00	2.60	0.33	55.63	28.49
	5	Ida V. Rautio	100.00	100.00	87.30	0.00	0.00	59.27	30.09

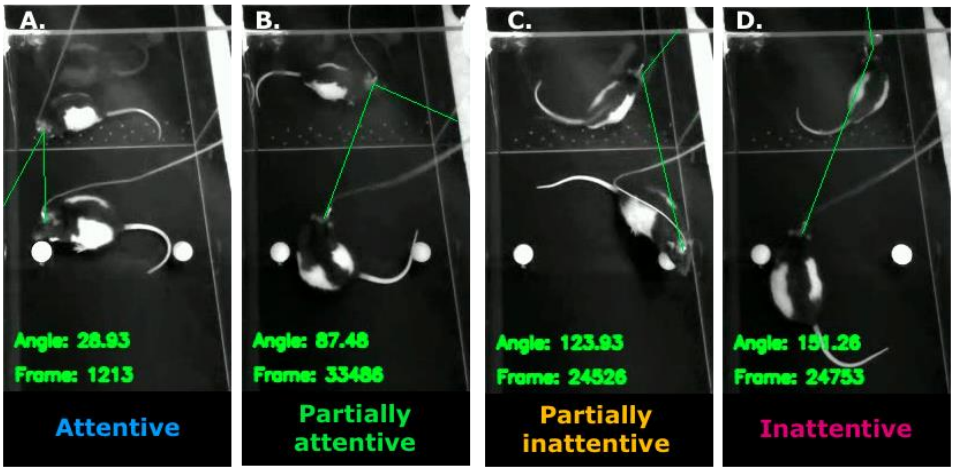
Supplemental data 3. Data from attention analysis

Supplementary Table C2: Example results of one video during protocol testing, where three random videos were selected to be compared after doing manual analysis on them. Analysis was done in three forms: all frame analysis of observer head angles with respect to performer (AF_PF); frames when task stimulus balls were ON and head angles calculated with respect to performer head (LO_PF) and stimulus balls (LO_SB). A:

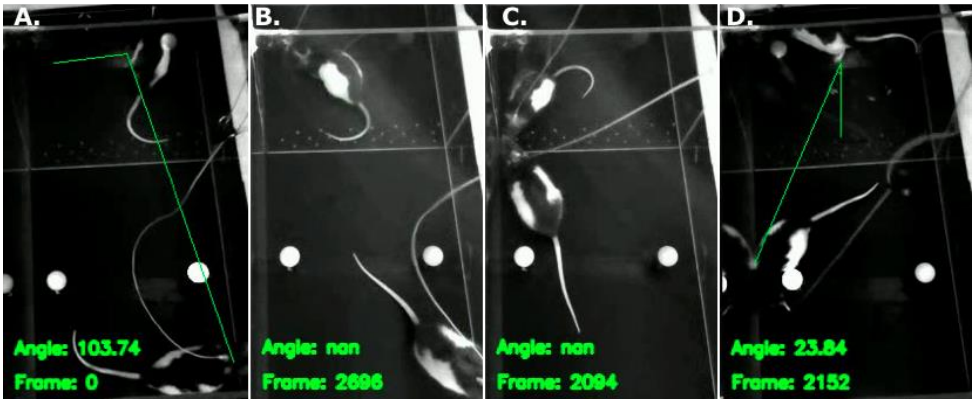
attentive, PA: partially attentive, PI: partially inattentive, and I: inattentive. LO_SB were not manually analyzed since it was established that observers' head angle with respect to stimulus ball was not relevant to social information, and hence, this thesis.

Protocol testing	Attention score of observer (%)											
	AF_PF				LO_PF				LO_SB			
	A	PA	PI	I	A	PA	PI	I	A	PA	PI	I
Video 1 (software)	27.34	48.40	10.97	13.29	28.69	46.51	10.32	14.48	28.98	48.19	10.78	12.05
Video 1 (manual)	43.48	25.52	10.64	20.36	30.60	32.27	12.72	24.41	–	–	–	–

Supplementary Figure C4: Example pictures of experiment chamber with performer and observer that exemplifies attentiveness behavior categorization during manual analysis.



Supplementary Figure C5: Demonstrates examples of incorrect head angle measurement by RAQ software.



Supplementary Table C3: Group attention scores calculated using csv. files produced by RAQ software with custom made MATLAB script (automated analysis) (Appendix A; Supplementary protocol 5). The scores were averaged across all data points within each of the groups: observation group (Obs; n=6), control group (Con; n=6), and ACC inhibited

group (ACC; n=5), for each of the three days of observational sessions (1 session per day). A: attentive, PA: partially attentive, PI: partially inattentive, and I: inattentive.

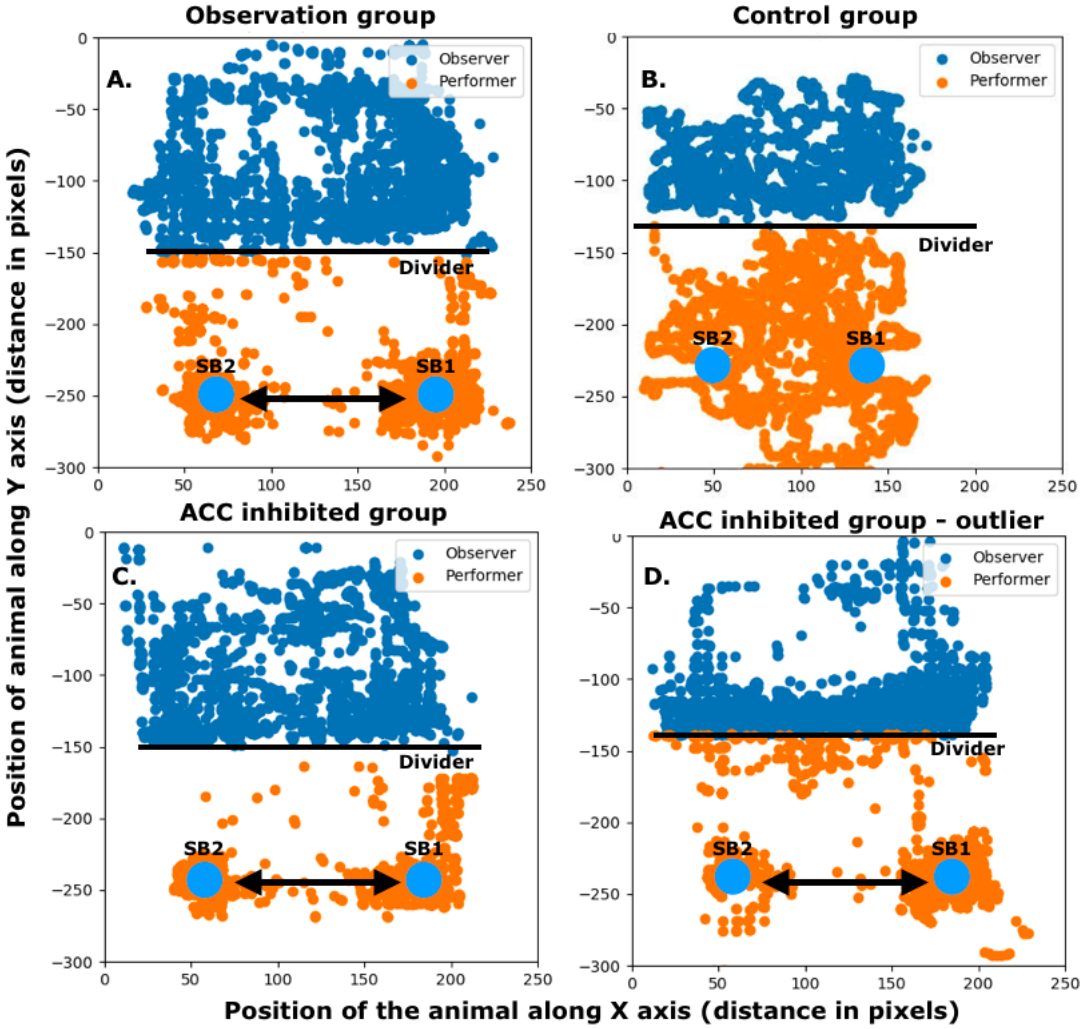
Automated	Day 1 to 3											
	Attention score of observer (%)											
	Day 1				Day 2				Day 3			
	A	PA	PI	I	A	PA	PI	I	A	PA	PI	I
Obs	27.73	49.27	10.08	12.93	28.74	48.89	9.86	12.52	28.18	49.40	9.63	12.80
Con	33.94	47.65	8.21	10.21	33.78	47.05	8.96	10.21	34.01	46.62	9.26	10.11
ACC	34.75	47.06	8.32	9.88	35.88	46.88	8.37	8.87	37.71	45.79	7.87	8.63

Supplementary Table C4: Individual attention scores calculated using csv. files produced by RAQ software with custom made MATLAB script. The scores were averaged across all three days of observational sessions (1 session per day) for each individual according to each of the groups: observation group (Obs; n=6), control group (Con; n=6), and ACC inhibited group (ACC; n=5). A: attentive, PA: partially attentive, PI: partially inattentive, and I: inattentive.

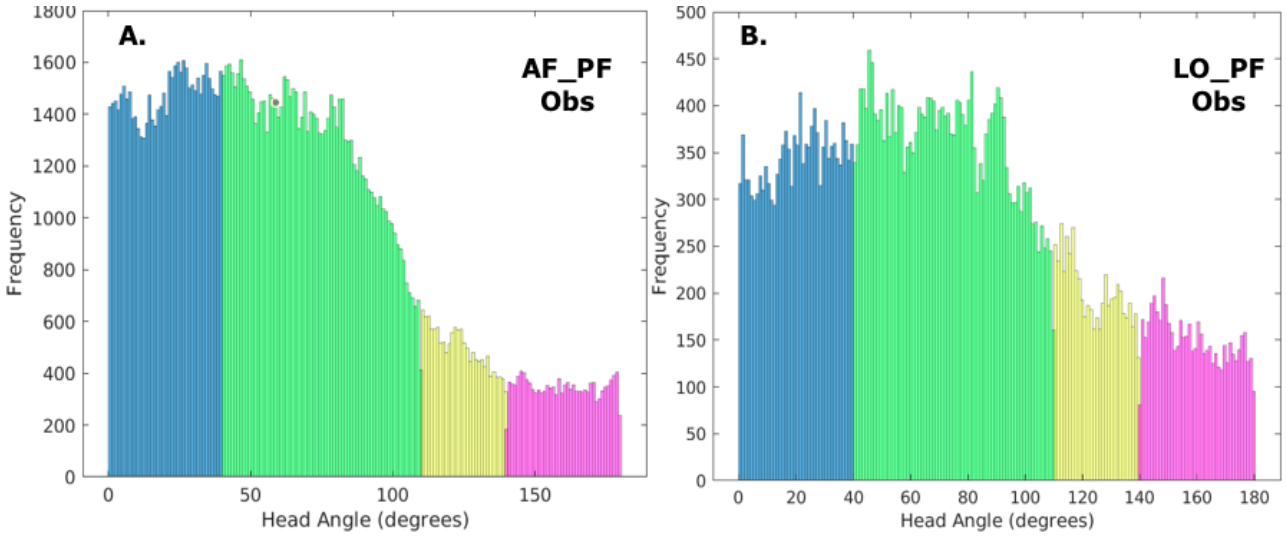
Experiment.	**No.**	**A**	**PA**	**PI**	**I**	**A+PA**	**I+PI**
Observational experiment	1	28.00	47.93	10.81	13.27	75.93	24.08
	2	24.04	47.42	11.99	16.55	71.46	28.54
	3	24.41	50.14	10.92	14.53	74.55	25.45
	4	28.98	50.61	8.86	11.55	79.59	20.41
	5	32.89	49.29	7.85	9.97	82.18	17.82
	6	30.98	49.72	8.68	10.62	80.7	19.3
Control experiment	1	29.50	47.88	10.75	11.87	77.38	22.62
	2	41.15	49.14	2.75	6.96	90.29	9.71
	3	38.86	47.44	6.43	7.27	86.3	13.7
	4	32.42	49.46	8.91	9.20	81.88	18.11
	5	31.32	44.01	11.90	12.76	75.33	24.66
	6	30.21	44.70	12.10	12.99	74.91	25.09
Observational experiment with ACC inhibition	1	30.90	49.45	8.61	11.04	80.35	19.65
	2	30.57	51.60	8.13	9.71	82.17	17.84
	3	32.72	47.58	10.26	9.44	80.3	19.7
	4	43.04	42.02	7.13	7.80	85.06	14.93
	5	43.33	42.22	6.80	7.65	85.55	14.45

Supplementary Figure C6: Position of observer (blue) and performer (orange) in the experiment box of LO_PF processed videos (divider is represented with a line). Points represent the position of the animal calculated using the location of the animal's snout for each frame. **Toggling behavior** was noticed when points representing performers position was grouped or crowded near the ball (represented with black line with arrows in A, C and D). This happened only when the animal had learned the task and could toggle between the balls as explained in Supplementary Figure C8. **A.** Example plot from day 2 of observational session for the observation group (trained performer), **B.** Example from day 2 of observational session from the control group (naive performer) where the

performer is exploring the chambers instead of performing (since no toggling is observed), **C.** Example plot from day 2 of observational session from the ACC inhibited group (trained performer), and **D.** Example plot from day 2 of observational session for observer 27260 from ACC inhibited group (the outlier). SB1/2: Stimulus ball.



Supplementary Figure C7: Illustrates the difference in head angle distribution of one observer animal (example taken from observation group; Obs) between two processing methods of RAQ software. **A.** Distribution of head angle over the entire 30 min session with respect to the performer's head position (all frames; AF_PF) **B.** Head angle distribution in processed videos (timestamped according to when stimulus ball was ON; LO) with respect to performers head (PF). Figures plotted using MATLAB (Appendix A; Supplementary protocol 5).



Supplementary Table C5: Group attention scores calculated using processed videos by automated and manual scoring of behavior (section 2.11.2.2). The scores were averaged across all data points within each of the groups: observation group (Obs; n=6), control group (Con; n=6), and ACC inhibited group (ACC; n=5) for each of the three days of observational sessions (1 session per day). A: attentive, PA: partially attentive, PI: partially inattentive, and I: inattentive.

Manual analysis	Day 1 to 3											
	Attention score of observer (%)											
	Day 1				Day 2				Day 3			
	A	PA	PI	I	A	PA	PI	I	A	PA	PI	I
Obs	41.09	18.58	14.75	25.58	47.22	24.18	11.37	17.24	49.86	15.15	13.12	21.87
Con	37.89	15.61	16.17	30.33	41.88	21.52	12.14	24.47	47.01	11.20	18.01	23.79
ACC	42.85	17.63	20.93	18.58	48.95	17.40	11.34	22.31	59.65	11.00	10.09	19.26

Supplementary Table C6: Individual attention scores calculated using processed videos by automated and manual scoring of behavior (section 2.11.2.2). The scores were averaged across all three days of observational sessions (1 session per day) for each individual according to each of the groups: observation group (Obs; n=6), control group (Con; n=6), and ACC inhibited group (ACC; n=5). A: attentive, PA: partially attentive, PI: partially inattentive, and I: inattentive.

Experiment.	No.	A	PA	PI	I	A+PA	I+PI
Observational experiment	1	39.92	25.46	8.98	25.64	65.38	34.62
	2	52.88	16.61	17.67	12.84	69.49	30.51
	3	55.04	11.95	12.84	20.17	66.99	33.01
	4	55.07	14.97	13.55	16.41	70.04	29.96
	5	26.96	34.27	16.81	21.96	61.23	38.77
	6	61.33	9.30	7.79	21.58	70.64	29.36
Control experiment	1	34.49	15.95	15.87	33.70	50.43	49.57
	2	46.86	11.39	17.21	24.53	58.25	41.75
	3	40.18	19.17	15.15	25.50	59.35	40.65
	4	49.14	6.75	15.36	28.74	55.89	44.11
	5	48.96	11.54	11.77	27.72	60.51	39.49
	6	33.91	31.85	17.26	16.98	65.76	34.24
Observational experiment with ACC inhibition	1	52.82	19.64	11.22	16.32	72.46	27.54
	2	48.39	14.28	12.70	24.63	62.67	37.33
	3	60.50	6.37	7.95	25.18	66.88	33.12
	4	53.61	9.94	18.23	18.23	63.54	36.46
	5	37.09	26.49	20.51	15.91	63.58	36.42

Supplementary Table C7: Shows correlation coefficients (rho) between attention score and performance of both performer (PF) and observer (OB) of control group. In this case, A: Attentiveness (A+PA); IA: Inattentiveness (I+PI); PST: Proportion of Successful Trials (performance, %); Significance measured at p <0.01.

		1.	2.	3.	4.
1.	Manual_A (%)	—			
2.	Manual_IA (%)	-1.00*	—		
3.	Automated_A (%)	-0.49	0.49	—	
4.	Automated_IA (%)	0.49	-0.49	-0.47	—
5.	Performance of observers (PST; %)	0.10	-0.10	-0.27	0.27

**Correlation is significant at the 0.01 level (2-tailed Spearman's rho)*

Supplementary Table C8: Shows correlation coefficients (rho) between, **A.** Attention scores of observers on day 1, day 2 and day 3 of all groups. The data used for this analysis is presented in supplementary Table C4 and C6. The scores were averaged across all data points within each of the groups: observation group (Obs; n=6), control group (Con; n=6), and ACC inhibited group (ACC; n=5) for each of the three days of observational sessions (1 session per day). **B.** Performance of the performer and performance and ATL of the observer in all groups. **C.** Performance of the performer and performance and ATL of the observer in Obs and ACC groups. Performance: Proportion of Successful Trials; ATL: Average Trial Length; PF: performer; OB: observer; TSET: time spent executing task (obtained from LO_PF processed videos). Significance measured at p <0.01 and p <0.05.

A.	All group	1.	2.	3.	4.	5.
1.	Day 1_Attentiveness	—				
2.	Day 1_Inattentiveness	-1.00**	—			
3.	Day 2_Attentiveness	0.50	-0.50	—		
4.	Day 2_Inttentiveness	-0.50	0.50	-1.00**	—	
5.	Day 3_Attentiveness	1.00**	-1.00**	0.50	-0.50	—
6.	Day 3_Inattentiveness	-1.00**	1.00**	-0.50	0.50	-1.00**

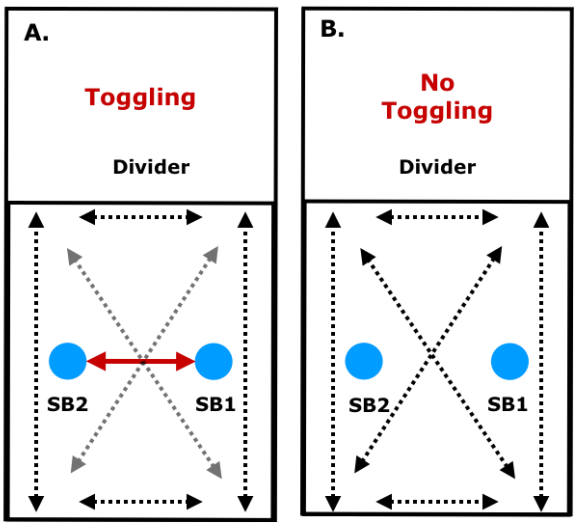
B.	All group	1.	2.	3.	4.
1.	PF_Performance (%)	—			
2.	PF_TSET (s)	0.27	—		
3.	OB_Performance (%)	-0.15	0.65*	—	
4.	OB_ATL (s)	0.164	-0.70*	-0.98**	—

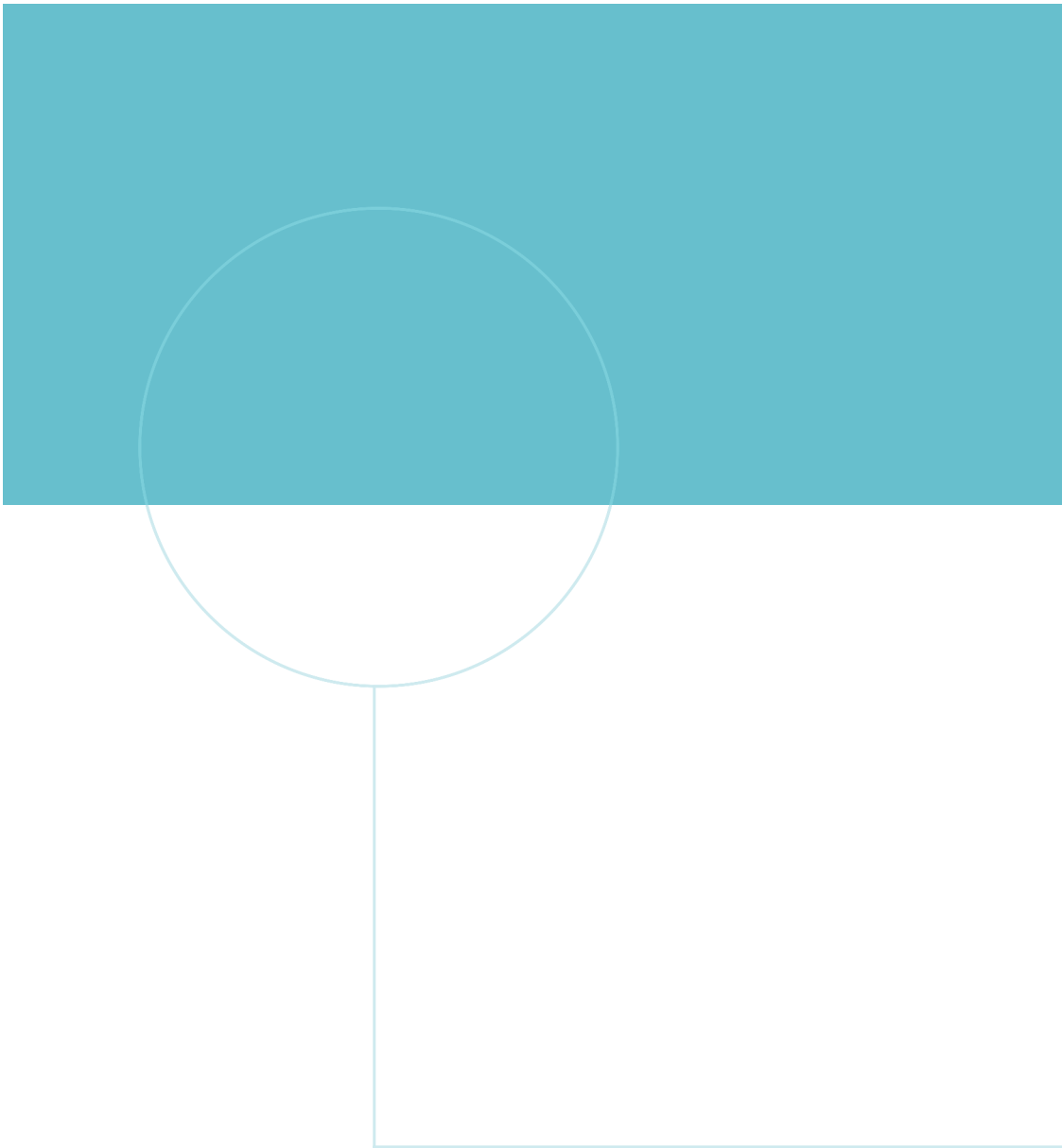
C.	Obs and ACC group	OB_ Performance (%)	OB_ALT (s)
	PF_TSET	0.07	-0.17
	PF_Performance (%)	0.28	-0.22

**Correlation is significant at the 0.05 level (2-tailed Spearman's rho)*
***Correlation is significant at the 0.01 level (2-tailed Spearman's rho)*

Supplementary Figure C8: Toggling behavior of a learned animal

Toggling behavior was defined by the movement of the performer from pushing SB1 to pushing SB2 in a strategic way such that rewarding stimulation was received at SB2. This pattern was only noticed in animals that had learned the stimulus ball pushing task explained in 2.4. Non-learned tend to explore the apparatus in all direction as shown in below in B, which sometimes lead to accidental pushing of the ball. **A.** Illustrates toggling behavior in performer camber on the testing day by observers that indicates learning. Continuous toggling was considered as an indication of learning (understanding the task). Red line: the path taken by the observer defined as toggling behavior, black dotted lines: other paths taken by the observer in the chamber (exploring). **B.** Represents usual paths taken by a non-learner, where toggling is missing.





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