The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

INVESTIGATING THE EFFECTS OF SILENCING THE HIPPOCAMPUS IN A PROBABILISTIC REVERSAL LEARNING TASK

submitted by Matthew Bannerman Cooke in partial fulfilment of the requirements for the degree of Master of Science in Neuroscience

Examinee Committee:

Jason Snyder, Associate Professor, Psychology, UBC

Supervisor

Stan Floresco, Professor, Psychology, UBC

Supervisory Committee Member

Yu Tian Wang, Professor, Neurology, UBC

Additional Examiner

Additional Supervisory Committee Members:

Mark Cembrowski, Assistant Professor, Cellular and Physiological Sciences, UBC
ABSTRACT

In the quest for rewards, which can range from food to other incentives, both animals and humans must navigate the inherent uncertainties of their environments. The ability to learn from and adapt to these uncertainties, modifying cognitive strategies for reward acquisition, is crucial. This adaptive process is compromised in various neurological and psychological disorders, including depression, obsessive-compulsive disorder (OCD), Parkinson’s disease, schizophrenia, among others. Traditionally, research has focused on the orbitofrontal cortex (OFC), striatum, and amygdala in forming probabilistic reward associations. Since the hippocampus is a brain region heavily involved in the formation and retrieval of memory, and probabilistic reversal learning is a learning task, we hypothesized that the hippocampus is involved. Emerging evidence, including prior research from our laboratory, suggests that hippocampal neurogenesis plays a role in modulating reward feedback sensitivity, hinting at a more nuanced involvement of the hippocampus in this process.

Given the hippocampus' extensive connections with the prefrontal cortex (PFC), amygdala, and striatum, we hypothesized that it might be a critical contributor to probabilistic learning mechanisms. The hippocampus itself is not a monolithic structure. It is differentiated into dorsal and ventral domains, with the dorsal primarily implicated in cognitive tasks such as spatial navigation and the ventral in encoding emotional significance. While historically considered in a dichotomous framework, contemporary studies indicate that both hippocampal regions engage in various learning and memory functions.
We employed both pharmacological and chemogenetic techniques to transiently inactivate the dorsal and ventral hippocampus. Pharmacological inactivation yielded observable effects in both regions. Chemogenetic inactivation of the dorsal hippocampus did not yield notable results. However, there were discernible and significant differences between the adeno-associated virus (AAV) treated and control groups. These findings may shed light on the differentiated, yet interrelated, roles of hippocampal regions in learning under uncertainty.

Our research demonstrates that pharmacological inactivation of the dorsal and ventral hippocampus lead to changes in perseverative behaviours, deemed Win-Stay, as well as impulsivity. Furthermore, chemogenetic inactivation shows diffuse effects of DREADD treatment versus non-surgical controls. AAV surgery leads to a decrease in performance measures (total reversals) as well as perseverative behaviour.
LAY SUMMARY

Learning and memory form a key part of human experience. Understanding how our brains form, retrieve, and store memories is vital for developing interventions that can improve or preserve cognitive functions. Learning and memory research can offer valuable insights into the rehabilitation of individuals with brain injuries or disabilities. Although the hippocampus plays an established role in many aspects of learning, understanding how the brain integrates statistical information, specifically through the hippocampus, is not well known. Probabilistic learning integrates statistical properties using uncertain events, where the subject must determine which strategy to use over many repeated learning experiences. Once the subject has developed a mental schema of the probable outcomes, they must use this knowledge to inform future actions. The goal of this research was to elucidate if the hippocampus plays a role in probabilistic learning. Our data suggest that there is involvement of the hippocampus in these behaviours.
PREFACE

This thesis contains unpublished original work by the author, Matthew Cooke under the supervision of Dr. Jason Snyder.

In Chapter 1, behavioural training and testing was performed Brie Dungate and Ricky Ma, alongside myself. Surgical interventions, tissue processing and data analyses were performed by myself.

In Chapter 2, Tyler Lin and myself performed animal behaviour. Surgical interventions, tissue processing and data analyses were performed by myself.

In Chapter 3, Likitha Mallela, Peyton Holder, and Si-ah Choi, as well as myself, participated in behavioural testing. Si-ah Choi and myself performed tissue processing and histological preparation. I performed data analyses.

Dr. Snyder provided critical guidance on project directions, methodology and data analysis.

Animal testing was conducted in accordance with the ethical guidelines of the Canada Council for Animal Care and were approved by the University of British Columbia Animal Care Committee under certificate A20-0290.
Work from Chapter 2 was presented at:
Society for Neuroscience meeting (2022)
DMCBH Research Retreat (2022)
Society for Neuroscience meeting (2023)

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Society for Neuroscience meeting (2023)


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# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>ACC</td>
<td>Anterior cingulate cortex</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CA</td>
<td>California</td>
</tr>
<tr>
<td>CA1</td>
<td>Cornu Ammonis area 1</td>
</tr>
<tr>
<td>CA2</td>
<td>Cornu Ammonis area 2</td>
</tr>
<tr>
<td>CA3</td>
<td>Cornu Ammonis area 3</td>
</tr>
<tr>
<td>CNO</td>
<td>clozapine N-oxide</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DC</td>
<td>District of Columbia</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate Gyrus</td>
</tr>
<tr>
<td>DMCBH</td>
<td>Djavad Mowafaghian Centre for Brain Health</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DREADD</td>
<td>Designer receptor exclusively activated by designer drugs</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated Plus Maze</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric Acid</td>
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<tr>
<td>GC</td>
<td>Genome-containing</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>MWM</td>
<td>Morris water maze</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>-------------------------------</td>
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<tr>
<td>NAc</td>
<td>Nucleus accumbens</td>
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<tr>
<td>OCD</td>
<td>Obsessive–compulsive disorder</td>
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<tr>
<td>OFC</td>
<td>Orbitofrontal cortex</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
</tr>
<tr>
<td>PRL</td>
<td>Probabilistic reversal learning</td>
</tr>
<tr>
<td>RFP</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>UBC</td>
<td>University of British Columbia</td>
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ACKNOWLEDGEMENTS

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I am also grateful for the guidance and assistance received from the Department of Neuroscience, which has been pivotal in navigating the challenges and milestones of this degree.
To my dog,

Hippo
Chapter 1: Introduction

1.1 The Hippocampus

Learning is defined as the acquisition of knowledge or skills through experiences, study, or teaching. It is a complex process that enables individuals to assimilate and apply new information. Memory, in contrast, is the process of retaining and recalling information, experiences, or skills that have been acquired through learning. This involves the storage and retrieval of information within the brain, making memory a fundamental component of learning and knowledge retention (Guskjolen & Cembrowski, 2023; Sridhar et al., 2023; Zlotnik & Vansintjan, 2019).

The hippocampus has long been implicated in the formation, consolidation, and retrieval of episodic memory (Gold & Squire, 2006; Squire, 2009). Episodic memory refers to the ability to remember specific events or experiences that occurred at a particular time and place, encompassing the personal experiences and the contexts in which they occurred (Eichenbaum, 2000; Wood et al., 2000; Zlotnik & Vansintjan, 2019). This type of memory allows individuals to recall past events, such as what they did last weekend or the details of a childhood birthday party. The relationship between the hippocampus and episodic memory is supported by a wealth of evidence from various research methodologies, including neuroimaging studies, neuropsychological assessments of individuals with hippocampal damage, and experimental studies in animals (Gothard et al., 1996; Kolibius et al., 2023; MacDonald et al., 2011; Voss et al., 2017).

One pivotal study was that of patient H.M. At the age of 27, H.M. underwent an experimental surgical procedure to alleviate severe epilepsy. The surgery, performed by
neurosurgeon William Scoville, involved the removal of large portions of H.M.'s medial
temporal lobes, including the hippocampus, amygdala, and adjacent cortical areas. While the
surgery succeeded in reducing his seizures, it resulted in profound anterograde amnesia,
rendering H.M. unable to form new long-term episodic memories, although his working memory
and procedural memory remained intact (Squire, 2009).

The case of H.M. became a pivotal study subject for neuropsychologist Brenda Milner
and others, who meticulously documented his cognitive deficits and capabilities over decades.
H.M.'s case provided clear evidence for the distinction between different types of memory,
specifically between episodic memory (the ability to recall specific events and experiences) and
procedural memory (the ability to learn tasks and skills). Despite his profound episodic memory
impairment, H.M. could learn and retain new motor skills, demonstrating that these types of
memory are supported by different neural circuits. H.M.'s condition underscored the crucial role
of the hippocampus in the consolidation of new episodic and semantic memories. His preserved
intellectual abilities and retention of memories formed before the surgery indicated that the
hippocampus is not the site of long-term memory storage, but is essential for forming,
organizing, and storing new memories. H.M. exhibited a temporal gradient in his retrograde
amnesia, with older memories being more intact than those formed closer to his surgery. This
observation contributed to the understanding of memory consolidation processes and the time-
dependent manner in which memories become less reliant on the hippocampus (Corkin, 2002;
Scoville & Milner, 1957).

Furthermore, the hippocampus plays a significant role in cognitive flexibility, defined as
the mental ability to switch between thinking about two different concepts or to think about
multiple concepts simultaneously (Buckner, 2010; Voss et al., 2011). Cognitive flexibility is a
crucial aspect of executive functions, enabling individuals to adapt their thinking and behavior in response to changing environments, rules, or demands. The involvement of the hippocampus in cognitive flexibility is primarily through its contributions to memory and learning processes, spatial navigation, and the integration of information, which are all essential for adapting to new situations and making decisions based on past experiences (Buckner, 2010; Eichenbaum et al., 1999; Maguire et al., 2006; Rubin et al., 2014).

1.1.1 The Trisynaptic Circuit

The trisynaptic circuit is a fundamental neural pathway within the hippocampus, integral to the process of memory formation. This circuit includes three key components: the dentate gyrus, the CA3 region, and the CA1 region (Figure 1). As information flows through this circuit, it is sequentially processed via excitatory synapses, which are crucial for the encoding and consolidation of declarative memories.

The first stage in the trisynaptic circuit is the dentate gyrus (DG). The DG is responsible for receiving sensory inputs from the entorhinal cortex and performing initial processing (Canto et al., 2008; Eichenbaum & Lipton, 2008). It plays a critical role in pattern separation, which helps in distinguishing between similar experiences or inputs (Treves & Rolls, 1994). Through adult neurogenesis, the continued integration of new neurons over the lifespan, the dentate gyrus is able to use a relatively small number of active neurons to represent information (Diamantaki et al., 2016). This sparse coding is believed to help encode new memories by transforming similar experiences and inputs into distinctly separate representations. In doing so, it is able to differentiate similar memories which we refer to as pattern separation.
The second stage, the CA3, is where the processed information from the DG is stored. The CA3 region is known for its auto-associative network, enabling the integration and association of different memory components (Bennett et al., 1994). It is essential for spatial memory and associative learning (Gilbert & Brushfield, 2009). These auto-associative recurrent connections are thought to allow for robust encoding, storage, and recall of memories. These recurrent collateral connections enable neurons in the CA3 region to activate each other and strengthen patterns of activity corresponding with memories. This process is called pattern completion.

In the final stage in the trisynaptic pathway, information from the CA3 region is transmitted to the CA1, which further processes and sends it to other brain regions, including back to the entorhinal cortex. The CA1 region is pivotal in the consolidation of long-term memory and is involved in the retrieval of spatial and contextual information (Bartsch et al., 2011). The CA1 acts as an interface between the hippocampus and other parts of the brain involved in memory processing, such as cortical regions. This area is essential for the long-term storage of memories and for navigating the process by which short-term memories are converted into long-term ones, a process known as memory consolidation. In addition to its role in memory consolidation, CA1 is also involved in memory retrieval. It helps in recalling specific memories in response to contextual cues, facilitating the process of reconstructing past experiences from stored information (Jimenez et al., 2020; Sans-Dublanc et al., 2020). This is particularly important for episodic memory, which involves recalling specific events or experiences that occurred at a particular time and place.

The efficiency and integrity of the trisynaptic circuit are vital for maintaining cognitive functions, particularly those related to memory. Studies in neurobiology and cognitive
neuroscience continue to explore the complexities of this circuit to better understand memory disorders and potential therapeutic interventions.

![Figure 1. Trisynaptic Circuit of the Hippocampus](image)

### 1.1.2 Dorsal Hippocampus

The dorsal hippocampus in rats is intricately connected with several key brain regions, playing a vital role in a range of cognitive functions. These connections are fundamental to the rat's ability to navigate space, consolidate memory, and regulate various cognitive processes.

The entorhinal cortex is a primary interface between the hippocampus and the neocortex. The connection with the entorhinal cortex is crucial for the processing and transmission of spatial and contextual information. It plays a significant role in the formation and retrieval of episodic and spatial memories (Basu & Siegelbaum, 2015). The perforant pathway connects the entorhinal cortex with the hippocampus.

The dorsal hippocampus interacts with the mPFC, a region involved in executive functions, decision-making, and working memory. This connection is key for integrating
memory and decision-making processes, particularly in tasks that require planning or strategy (Eichenbaum, 2017).

The septum, which connects with the dorsal hippocampus, plays an essential role in modulating the activity of hippocampal neurons. This connection is particularly important for the regulation of the theta rhythm, a brain wave pattern associated with navigation, memory formation, and attention (Colgin, 2016; Müller & Remy, 2018).

These connections underscore the dorsal hippocampus’ role in higher-order cognitive processes. The entorhinal cortex and septum connections facilitate spatial and memory functions, while its interaction with the mPFC is key for complex cognitive tasks (Palombo et al., 2019). Understanding these pathways is crucial for deciphering the neural basis of learning, memory, and behavior in rats, and can also provide insights relevant to human neurobiology.

1.1.3 Ventral Hippocampus

The ventral hippocampus in rats plays a crucial role in the limbic neural network, establishing connections with several key brain regions that contribute to a wide range of physiological functions.

The ventral hippocampus’ connection to the nucleus accumbens (NAc) is pivotal for reward processing and motivational behaviors. The nucleus accumbens is a core component of the brain's reward circuit, and its interaction with the ventral hippocampus is essential in forming reward-related memories and guiding motivated behaviors (LeGates et al., 2018).

The linkage between the ventral hippocampus and the amygdala is fundamental in regulating emotional responses and processing. This connection is particularly important for fear
learning and the emotional components of memory, playing a significant role in how rats respond to stressful or threatening stimuli (Yang & Wang, 2017).

The ventral hippocampal-hypothalamic circuit is crucial for the regulation of stress responses and homeostatic processes. These hypothalamic projections to the ventral hippocampus have been shown to be implicated in impulsive and motivated responding (Noble et al., 2019). The hypothalamus is central to the body's response to stress and is involved in various autonomic functions (Gergues et al., 2020).

Projections to the prefrontal cortex are key in higher-order cognitive functions. The prefrontal cortex is involved in decision-making, planning, and moderating social behavior. The connection with the ventral hippocampus integrates emotional and cognitive processes, influencing how rats assess and respond to their environment (Sun et al., 2020).

These diverse connections highlight the ventral hippocampus' significant role in orchestrating complex behaviors and physiological responses in rats. It is not only involved in fundamental emotional processes, but also plays a role in integrating these processes with cognitive functions. This illustrates the intricate interplay between different brain regions in shaping behavior and physiological responses.

Furthermore, the ventral hippocampus has more recently been shown to play a role in spatial navigation. Rodent studies with ventral hippocampal disruption have shown spatial navigation deficits in the Morris water maze, although with less impact than dorsal lesions (Loureiro et al., 2012). The ventral hippocampus has also been shown to play a role in navigation where the goal location determines reward magnitude (Garvert et al., 2023).

Overall, the ventral hippocampus has been shown to play a role in many different aspects of behaviour. Its utility in reward processing and navigation highlights its possible contributions
to multiple aspects of the probabilistic reversal learning (PRL) task. This cognitive task necessitates the dynamic updating of reward salience, the strategizing of future actions, and responses to unforeseen outcomes. Additionally, spatial mapping of reward contingencies and their flexible updating could be key to the flexible behaviour involved in reversals in this task. Consequently, it is hypothesized that the ventral hippocampus is instrumental in executing the PRL task, supporting the neural processes underlying adaptive decision-making and learning from feedback. This hypothesis is grounded in the ventral hippocampus's established contributions to the integration of emotional and contextual information, which are critical for evaluating and revising behavioral strategies in response to changing environmental contingencies.

1.1.4 Consolidating distinct regions

While both the dorsal and ventral hippocampus in rats are involved in regulating various behaviors, they exhibit distinct functional specializations. The dorsal hippocampus is more closely linked to cognitive processes and spatial memory, whereas the ventral hippocampus is commonly associated with emotional regulation, stress responses, and reward-related behaviors (Table 1). These distinctions reflect the anatomical and functional heterogeneity within the rat hippocampus.
### Table 1. An overview of dorsal and ventral hippocampus.

<table>
<thead>
<tr>
<th>Aspect</th>
<th>Dorsal Hippocampus</th>
<th>Ventral Hippocampus</th>
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<tbody>
<tr>
<td><strong>Function</strong></td>
<td>• Spatial memory • Episodic Memory</td>
<td>• Emotional regulation • Stress Response • Reward-related behaviours</td>
</tr>
<tr>
<td><strong>Connections</strong></td>
<td>• Entorhinal cortex • Septum • Retrosplenial cortex • Anterior cingulate cortex</td>
<td>• Olfactory bulb • Amygdala • Infra-limbic, pre-limbic cortex • Hypothalamus • Medial prefrontal cortex</td>
</tr>
</tbody>
</table>

However, recent evidence suggests a potential homogeneity between these regions, with the ventral hippocampus possibly storing broader representations of spatial information (Loureiro et al., 2012; Muller et al., 1994; Strange et al., 2014).

### 1.2 Other forms of learning and memory

#### 1.2.1 Statistical learning

Statistical learning is an implicit cognitive process where individuals subconsciously extract regularities, patterns, or statistical properties from their environment through repeated exposure to stimuli (Sherman et al., 2020). This form of learning occurs without conscious awareness and is primarily linked to the ability to discern and predict relationships between elements in a sequence, whether they be sounds, visual stimuli, or other patterns, by recognizing
the statistical regularities present (Turk-Browne et al., 2009). For example, through repeated experiences, you may learn that a certain route to work often takes longer than another and you may adapt your route in preference of the shorter option. You continue to subconsciously update this representation as you travel to work and flexibly adapt your navigation depending on the outcomes.

In statistical learning, the brain uses statistical information to form predictions about upcoming events. This mechanism is crucial across various domains, particularly in language acquisition (Williams, 2020). In this context, individuals unconsciously absorb the regularities and structures of a language through exposure to spoken or written words. Statistical learning is, therefore, considered a vital component of human learning and cognition, shaping our understanding of the world.

The striatum, a key component of the basal ganglia, is deeply involved in statistical learning, acting as a critical neural substrate for this process (Orpella et al., 2021). The involvement of the striatum in statistical learning underscores its broader role in habit formation, decision-making, and the reinforcement learning mechanisms. The striatum receives dopaminergic inputs from the midbrain, which are crucial for modulating learning processes and reward-related signals. During statistical learning, the striatum is thought to contribute to the detection and reinforcement of regularities and patterns that are encountered, integrating sensory inputs with reward information to reinforce the prediction of outcomes based on past experiences (Shohamy, 2011).

Research has shown that the striatum is particularly active when individuals are engaged in tasks that require the recognition of patterns and the learning of probabilistic sequences, whether in visual, auditory, or motor domains (Orpella et al., 2021; Shohamy, 2011; Turk-
Browne et al., 2009; Yin & Knowlton, 2006). This activity suggests that the striatum is involved in tracking the occurrence and co-occurrence of elements, facilitating the learning of complex structures through its connections with various cortical areas. For example, in language learning, the striatum supports the acquisition of grammar and syntax by detecting and reinforcing the statistical properties of linguistic inputs (Ripollés et al., 2014).

Moreover, the striatum's role in statistical learning is closely linked to its involvement in the reward system. It evaluates the outcomes of actions and the salience of stimuli, which is fundamental for adjusting behavior based on the statistical properties of the environment (Orpella et al., 2021; Ripollés et al., 2014; Shohamy, 2011). This connection between statistical learning and reward processing illustrates how adaptive behavior is shaped by the ability to learn from the statistical structure of the environment, with the striatum playing a pivotal role in these learning and adaptation processes.

Other research has shown that the hippocampus, a brain region integral to learning and memory, is involved in statistical learning from infancy (Ellis et al., 2021). Infants display an ability for statistical learning, implicitly detecting and learning patterns and regularities in their environment. This capability has been observed in studies focusing on both auditory and visual stimuli. For example, infants who are only around three months old have demonstrated the ability to learn statistical regularities in speech sounds or visual sequences and correlated with the hippocampal formation in fMRI (Ellis et al., 2021).

The role of the hippocampus in the encoding and processing of statistical information is a key focus in understanding the mechanisms of learning and memory. Its involvement from such an early age underscores the fundamental nature of statistical learning in cognitive development. This research provides valuable insights into how humans, even in infancy, begin to process and
understand the complex world around them. The hippocampus has also been shown to be involved in statistical learning later in life. A sample of adult patients with hippocampal damage showed lower ability to learn statistical information (Covington et al., 2018). Furthermore, medial temporal lobe damage, including the hippocampus, was implicated in ablation of statistical learning in a human case study (Schapiro et al., 2014).

Despite the recognition of the hippocampus’ role in statistical learning, there is limited research exploring which aspects of statistical learning the hippocampus is critical for. To address this gap, we have employed a rodent model, utilizing a probabilistic reversal task. This task is designed to assess the involvement of the hippocampus in learning probabilistic values and its capacity to flexibly update these representations over time.

1.2.2 Probabilistic reversal learning

In order to study statistical learning, tasks using non-deterministic outcomes had to be developed. These probabilistic reversal learning tasks are utilized to study cognitive flexibility and decision-making processes (Shohamy et al., 2009).

Early investigations into human cognitive processes have extensively employed probabilistic reversal learning tasks to examine the mechanisms underlying stimulus-reward associations. Such tasks, notably Dr. Trevor Robbins' probabilistic reversal learning task and the weather prediction task developed by Knowlton et al., are designed to simulate real-world decision-making scenarios where outcomes are uncertain and based on probabilistic cues.

In Robbins' probabilistic reversal learning task, participants are presented with stimuli that are associated with rewards or penalties based on certain probabilities. The task requires participants to learn and adapt to these probabilities to optimize their choices. This paradigm is
pivotal in understanding the neural mechanisms of feedback processing and decision-making, particularly in how individuals adjust their behaviors in response to changing reward contingencies (Cools et al., 2002).

Similarly, the weather prediction task by Knowlton et al. involves participants learning to predict weather outcomes (e.g., rain or sunshine) based on a series of probabilistic cues. This task has been instrumental in studying the role of the basal ganglia and the hippocampus in probabilistic learning and the distinction between declarative and procedural memory systems (Knowlton et al., 1994).

These tasks underscore the complexity of human decision-making, highlighting how our brains process and integrate multiple streams of probabilistic information to guide behavior. The probabilistic nature of these tasks mirrors the uncertainties inherent in real-life decision-making, providing valuable insights into the cognitive and neural mechanisms that support adaptive behavior in dynamic environments. However, the human literature has not shown clear hippocampal involvement in these tasks (Duncan et al., 2018; Foerde & Shohamy, 2011).

Probabilistic reversal learning tasks in rats serve as valuable tools in exploring the neural circuits and mechanisms that underpin cognitive flexibility, decision-making under uncertainty, and the capability to adjust to changing environments. Work by Dr. Stan Floresco has significantly contributed to our understanding of decision-making processes, particularly those involving risk, reward, and the ability to adapt to changing contingencies. One key aspect of Dr. Floresco's work is the exploration of how different brain regions contribute to the assessment of risk and reward, and how they enable animals to update their choices in response to changing probabilities of outcomes (Dalton et al., 2014, 2016; Floresco, 2013; Jenni et al., 2021; J. D. Schumacher et al., 2021; Seib et al., 2021). This involves not just the striatum, but also intricate
interactions between the orbitofrontal cortex, which is involved in evaluating the value of different outcomes, and the dorsal striatum, which is crucial for habit formation and action selection.

Using the probabilistic reversal learning task Dr. Floresco has developed, our lab has found evidence suggesting hippocampal involvement in this process. Preliminary research in our laboratory has indicated that adult neurogenesis – the integration of newborn neurons into the dentate gyrus of the hippocampus later in an animal's life – plays a role in probabilistic reversal learning (Seib et al., 2020). Notably, animals lacking adult neurogenesis demonstrated impairments in lose-shift behaviors on the correct lever, and win-stay behaviors on the incorrect lever. Additionally, neurogenesis-ablated animals took longer to reverse their choices in the early stages of the trials.

The implications of these findings extend to human health outcomes. Win-stay behaviors, where an animal returns to a previously rewarded lever on the subsequent trial, can be interpreted as the ability to utilize reward information from a recent trial to guide current behavior, while lose-shift behaviors, where an unrewarded animal switches levers, may serve as a proxy for sensitivity to negative feedback. Individuals with depression often exhibit alterations in their sensitivity to negative feedback, contributing to the characteristic cognitive and emotional patterns associated with the disorder (Mueller et al., 2015). This highlights the potential relevance of these animal models in understanding and addressing human mental health issues.

Our study also employs a reversal learning paradigm to assess behavioral flexibility. In this paradigm, once an animal has mastered a specific response based on given contingencies, these contingencies are altered, necessitating an adjustment in the animal's response strategy.
This approach not only probes the capacity for behavioral adaptation, but also allows for the exploration of the neural underpinnings associated with this flexibility.

Behavioral flexibility is a critical cognitive function that enables individuals to adapt to changing environments and modify behaviors based on new information. Unlike learning from isolated events, behavioral flexibility involves the integration and re-evaluation of accumulated experiences, necessitating the ability to adjust to new circumstances seamlessly. This cognitive flexibility is believed to be closely linked with the hippocampus (Rubin et al., 2014).

However, research indicates that behavioral flexibility is not solely dependent on the hippocampus. It also involves a network of brain regions. For instance, studies have highlighted the prefrontal cortex's involvement in executive functions, including decision-making and inhibitory control, which are essential for adaptive behavior in changing environments (Floresco, 2013). Moreover, the basal ganglia, including the striatum, have been implicated in habit formation and the modulation of actions based on reward outcomes, playing a significant role in revising previously learned behaviors (Yin & Knowlton, 2006).

Therefore, while the hippocampus contributes to behavioral flexibility, particularly in tasks requiring the integration of spatial and contextual information, it is part of a broader neural circuitry that orchestrates this complex cognitive ability. Understanding the dynamic interactions among these regions is crucial for elucidating the mechanisms of adaptive behavior and may offer insights into disorders characterized by cognitive rigidity and impaired adaptive learning.

1.2.3 Non-hippocampal circuits involved in probabilistic learning

Probabilistic reversal learning involves several distinct regions of the brain, each playing a critical role in processing and decision-making. The orbitofrontal cortex (OFC) is pivotal in
evaluating expected outcomes and adapting behavior based on changing reward contingencies. The striatum, particularly the caudate nucleus, is involved in the formation and updating of action-outcome associations, contributing to the selection of advantageous choices. The anterior cingulate cortex (ACC) also plays a crucial role, particularly in monitoring performance, detecting errors, and signaling the need for behavioral adjustments. Additionally, the prefrontal cortex (PFC) is implicated in maintaining and shifting cognitive sets, essential for adapting to reversal contingencies. These regions collectively contribute to the complex cognitive processes underlying probabilistic reversal learning, integrating feedback, and guiding adaptive behavior in changing environments. This understanding is supported by neuroimaging studies and lesion analyses, which reveal how disruptions in these areas can impact the ability to learn from probabilistic feedback.

The hippocampus, while primarily known for its role in memory formation and spatial navigation, might also be involved in probabilistic reversal learning due to its extensive connections with the aforementioned brain regions.

1.2.4 Possible Hippocampal involvement in probabilistic reversal learning

The hippocampus is integral in forming and retrieving memories, particularly contextual and episodic memories. In probabilistic reversal learning, the ability to remember past outcomes and contexts of decisions is crucial. The hippocampus may contribute to this process by integrating past experiences and outcomes with current decision-making processes.

There is a significant interaction between the hippocampus and the prefrontal cortex (PFC). This connection is crucial for integrating working memory and contextual information in decision-making, skills essential in probabilistic reversal learning where adapting to changing
rules or environments is necessary. It also projects to the striatum, which is involved in habit formation and reward processing. This interaction could play a role in modifying behavior based on the changing reward structures in probabilistic reversal learning tasks. The hippocampus, through its connections with the anterior cingulate cortex (ACC), may contribute to error detection and the adjustment of behaviors, which are central to mastering reversal learning tasks. The hippocampus has been shown to be involved in regulating stress and emotional responses, which can influence cognitive processes like decision-making and learning. In tasks that require adaptation to changing conditions, such as probabilistic reversal learning, the individual’s stress response can significantly affect performance (Zhang et al., 2020).

Overall, while the hippocampus is not the primary region associated with probabilistic reversal learning, its extensive connections with key brain regions involved in this process suggest that it can play a supportive role in integrating memory and contextual information, contributing to effective decision-making, and learning in changing environments.

1.3 Research Question

Which subregions of the hippocampus, if any, are involved in probabilistic reversal learning, and which aspects of the animal’s behaviour do they support.
Chapter 2: Inhibition of Dorsal and Ventral Hippocampus using GABA agonists in Probabilistic Reversal Learning

2.1 Introduction

The investigation into which subregions of the hippocampus are implicated in probabilistic reversal learning, and the specific aspects of animal behavior they influence, is a nuanced area of neuroscience research. Current evidence suggests differential involvement of hippocampal subregions in this learning process, each supporting distinct facets of behavior.

The dorsal hippocampus, primarily associated with cognitive functions and spatial memory, plays a crucial role in the encoding and retrieval of spatial information, a key component in probabilistic reversal learning. Studies indicate that the dorsal hippocampus supports an animal’s ability to navigate and adapt to changing spatial contingencies, a fundamental aspect of the reversal phase of learning (Kesner, 2007; Lee et al., 2005).

Conversely, the ventral hippocampus, known for its involvement in emotional regulation and stress responses, contributes to the affective aspects of learning and decision-making. This includes managing anxiety and stress that may arise due to changing environmental conditions or shifting reward probabilities (Fanselow & Dong, 2010; Moser & Moser, 1998). For instance, research has shown that the ventral hippocampus modulates responses to uncertainty and reward prediction errors, which are integral to probabilistic reversal learning tasks (Kheirbek et al., 2013). Furthermore, the ventral hippocampus’ established role in the emotional salience of experiences and reward processing could play a key role in the updating of reward
representations during behaviour (Komorowski et al., 2013; Riaz et al., 2017a, 2017b; A. Schumacher et al., 2016).

Moreover, recent studies have begun to explore the role of intermediate hippocampal regions. The research suggests that these areas might serve as integrative zones for cognitive and emotional information processing, thereby facilitating more complex decision-making behaviors in probabilistic tasks (Strange et al., 2014).

The involvement of specific hippocampal subregions in probabilistic reversal learning underscores the hippocampus’ multifaceted role in both cognitive and emotional aspects of learning and behavior. This complexity not only enriches our understanding of hippocampal functions but also highlights the region’s significance in adaptive learning and behavior modulation. In order to investigate the hippocampus’ contributions to these behaviors, direct inactivation of specific brain regions is required. By using pharmacological agents to silence specific hippocampal subregions, we can investigate the roles these regions play in our probabilistic reversal learning task.

Muscimol and baclofen have been widely utilized in neuroscientific research as pharmacological agents for the targeted inactivation of specific brain regions, a method crucial for dissecting the functional contributions of distinct neural circuits to behavior and cognition. Muscimol acts as a potent agonist of the GABA\textsubscript{A} receptors, while baclofen activates GABA\textsubscript{B} receptors, both leading to hyperpolarization of neurons and effectively reducing their activity (Bowery et al., 2002; Martin & Ghez, 1999).

GABA (gamma-aminobutyric acid) is the primary inhibitory neurotransmitter in the brain. Agonists of GABA receptors, such as GABA\textsubscript{A} and GABA\textsubscript{B}, enhance the inhibitory effects
of GABA, leading to a reduction in neuronal excitability. In the context of the hippocampus, this reduction in excitability translates to a temporary silencing of hippocampal activity.

This silencing effect is critical for isolating and understanding the specific contributions of the hippocampus to learning processes. In the case of probabilistic reversal learning, where the ability to adapt to changing reward contingencies is essential, the inactivation of the hippocampus allows researchers to observe how its absence affects learning and decision-making behaviors. This, in turn, helps in delineating the functional role of the hippocampus and its subregions in complex cognitive tasks.

This pharmacological inactivation allows us to observe the behavioral and physiological outcomes of silencing particular areas of the brain, providing insights into the roles these regions play in various cognitive processes, such as learning, memory, and emotional regulation. The precision and reversibility of the effects induced by muscimol and baclofen have made them indispensable tools in behavioral neuroscience, facilitating the exploration of neural circuitry dynamics by offering a transient, controlled disruption of normal neural activity without causing permanent damage to the tissue (Duvarci & Pare, 2014). In this study, we employ this paradigm to investigate the role of dorsal and ventral hippocampus in probabilistic reversal learning.

2.2 Methods

2.2.1 Animals

16 Long-Evans rats were utilized for this experiment. Eight male and female rats were acquired from Charles-River laboratories at 8 weeks of age. Prior to any behavioral assessments, these animals were acclimated to handling by all experimenters to mitigate any stress associated
with handling during the experiments. Throughout the behavioral training and testing phases, the animals were subjected to food restriction.

The animals were housed in pairs until the time of surgery, after which they were housed individually. Animals were initially habituated to their home-cage upon arrival to our animal facility. All rats were initially trained to press levers in operant chamber for a 40mg sucrose reward. Once lever-pressing was conditioned to the reward, animals were trained on the probabilistic reversal learning task. After training, animals underwent surgery to implant bilateral stainless steel cannula into either dorsal (4 males, 4 females) or ventral (4 males, 4 females) hippocampus. Animals were assigned to receive either dorsal or ventral cannula randomly, with 4 males receiving dorsal and 4 females receiving ventral hippocampal cannula. Animals were given 2 weeks to recover from surgery before being retrained on the PRL task. Animals were habituated to the infusion process and then tested in 2 sessions, receiving either vehicle or muscimol/baclofen. Following testing, animals performed the elevated plus maze and Morris water maze. Animals were euthanized immediately after the Morris water maze to prevent infection through the cannula. Due to high rates of infection from the open cannula in the post-surgical period, 5 animals had to be euthanized prior to testing. Our behavioural data are presented with 4 ventral (1F, 3M), and 6 dorsal (2F, 4M) animals (N=10).

All animal work was done in accordance with UBC’s animal care regulations.

2.2.2 Food restriction

Throughout the experiment, all rats were subjected to food restriction, maintaining their body weight at approximately 85% of their ad libitum (A.L.) weight. This weight management was closely monitored in accordance with a Charles River growth chart. During any surgical
intervention and the subsequent recovery phases, the animals were returned to ad libitum feeding to support their health and recovery. The specific weights of individual animals, as recorded throughout the experiment, are detailed in (Figure 2) below. This table provides a comprehensive overview of the weight management protocol applied to each animal during the study.

![Figure 2. Weights of the animals plotted over the experiment. Animals under food restriction stabilised early in the experiment and continued to gain weight. Females plateau before males.](image)

### 2.2.3 Probabilistic reversal learning task

To investigate probabilistic reversal learning in a rodent model, a probabilistic reversal operant task was employed, utilizing operant chambers (30.5 cm × 24 cm × 21 cm) from Med Associates (Bari et al., 2010; Dalton et al., 2014). These chambers were equipped with left and right retracting levers, a solid reward hopper, and lights. The task presented the rats with both levers, designating one as the high-reward (correct) lever with an 80% reward probability, and the other as the low-reward (incorrect) lever with a 20% reward probability. These lever
contingencies remained constant until the rat successfully identified and responded to the high-reward lever for eight consecutive trials, triggering a contingency reversal (Figure 3).

Each daily session comprised 200 trials for each animal. At the start of an active trial, the chamber lights were activated and both levers extended. A trial concluded either after the animal's response or after an omission, defined as a 10-second period without a response, following which the levers retracted and the house light turned off until the next trial. The total duration of the task was set at 55 minutes, with all training sessions conducted once daily during the light phase of the light-dark cycle. Upon receiving a reward, a 40mg sucrose pellet was dispensed into the food hopper.

Behavioral data from these sessions were recorded and exported using Med Associates’ “MED-PC” and “MED-PC to Excel” software, respectively. This data collection method ensured accurate and comprehensive recording of the rats' performance throughout the task.

Over the duration of the probabilistic reversal learning task we analyzed the behaviours outlined in Table 2. These analyzed behaviours represent key aspects of task performance.
<table>
<thead>
<tr>
<th>Behaviour</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reversal</td>
<td>After 8 consecutive correct choices, the lever contingencies switch. This is deemed a reversal.</td>
</tr>
<tr>
<td>Correct Press</td>
<td>A response on the high-rewarding lever.</td>
</tr>
<tr>
<td>Win-Stay</td>
<td>A consecutive response on the same lever after receiving a reward.</td>
</tr>
<tr>
<td>Lose-Shift</td>
<td>Switching to the other lever after not being rewarded on the previous trial.</td>
</tr>
<tr>
<td>Omission</td>
<td>No response during the trial. Inverse of lever press.</td>
</tr>
<tr>
<td>Lever Press</td>
<td>A press of either lever. Inverse of an omission.</td>
</tr>
<tr>
<td>Latency</td>
<td>The time between the start of the trial and a lever-press is recorded.</td>
</tr>
</tbody>
</table>

Table 2. List of analyzed behaviours

2.2.4 Cannula

Bilateral custom-made cannulas (23-gauge, stainless steel) were surgically implanted to target specific areas of the rat brain. For targeting the dorsal hippocampus, cannulas were positioned at coordinates 3.8mm posterior, 2.5mm lateral to bregma, and 2mm ventral to the dura (which is 0.5mm dorsal to the target site). To target the ventral hippocampus, the cannulas were implanted at 5.2mm posterior, 5.4mm lateral to bregma, and 5.5mm ventral to the dura. The accuracy of these coordinates was confirmed stereotaxically.
The surgical procedures were conducted under isoflurane anesthesia, adhering to stereotaxic techniques. Post-surgery, animals were administered meloxicam and bupivacaine for pain relief.

Prior to testing, the animals were infused with 0.5μl of a solution containing muscimol and baclofen (GABAa and GABAb agonists, respectively) to inhibit activity in the targeted brain region, or they received a vehicle solution (0.9% saline, 0.5μl). This infusion was administered at a rate of 0.25μl/minute using an infusion pump. To ensure adequate diffusion of the drug cocktail, infusion needles remained in place for an additional minute following the infusion. The rats were then returned to their home cages for a 20-minute period before the commencement of the operant test. All animals were infused with both muscimol/baclofen and vehicle in a within-subjects design with 2 days rest between test sessions. Order of infusion was randomly chosen and counterbalanced between animals.
2.2.5 Control tasks

To confirm that animals receiving hippocampal inactivation exhibited behavioral effects, animals underwent behavioural control tasks. Animals with dorsal cannulas were tested using the Morris Water Maze (MWM), a well-established assessment of spatial navigation that depends on the dorsal hippocampus. Animals with ventral hippocampal inactivation perform the elevated plus maze (EPM), a test for anxiety behaviours reliant on ventral hippocampal activity. Animals with dorsal cannula received vehicle infusions (0.9% saline, 0.5μl) and acted as a control group.
in the EPM task. Animals with ventral cannula received vehicle infusions and acted as controls in
the MWM task.

In the MWM, animals were required to locate a hidden, submerged platform within a 2-
meter diameter pool filled with 22°C water, which was rendered opaque using non-toxic paint
(Figure 6). Each animal was allotted 60 seconds per trial to find the hidden platform. If an animal
failed to locate the platform within the allotted time, the experimenter would guide it to the
platform to facilitate learning. The sessions consisted of 8 trials per animal.

Behavioral data was captured using Anymaze tracking software and subsequently
analyzed with the Pathfinder software package specifically designed for the Morris Water Maze
(Cooke et al., 2019). This approach allowed for a thorough evaluation of the spatial navigation
abilities of the animals, post-hippocampal inactivation (Figure 5). Latency and swim path length
were also analyzed. Animals were euthanized immediately after the final Morris water maze
trial.
Figure 5. Search Strategy examples for Morris Water Maze analysis with definitions of the parameters for each strategy.

Animals equipped with ventral cannulas were evaluated using the Elevated Plus Maze (EPM) test. The EPM consists of a raised platform featuring four outstretched arms and a central region. Two of these arms are enclosed (closed arms), while the remaining two are open (Figure 6). To induce anxiety-related behaviors, room lights were kept at their brightest level during the trials.

Each trial in the EPM lasted 10 minutes per animal. The movements and behaviors of the animals were monitored using a ceiling-mounted camera. The behaviors observed in the maze were then identified and recorded manually. This setup and methodology were designed to assess the anxiety-related behaviors of the animals, providing insights into the effects of ventral hippocampal inactivation.
Figure 6. A. A schematic of the Morris Water Maze with the escape platform in the North-East quadrant. B. A Schematic of the Elevated Plus Maze with open (North, South) and closed (East, West) arms.

### 2.2.6 Tissue collection

Immediately after euthanasia, the brains of the rats were carefully extracted and fixed in a 4% paraformaldehyde solution (PFA). The fixation process using PFA is essential for preserving the structural integrity of the brain tissue, which is critical for subsequent analytical procedures. Paraformaldehyde fixation stabilizes the tissue architecture by cross-linking proteins, thereby maintaining the cellular and subcellular structures intact for detailed examination.

For the purpose of tissue sampling, a vibratome, an instrument designed for precision slicing of tissue, was employed. The fixed brain tissue was sectioned into slices with a thickness of 150 μm each. This specific slice thickness was chosen to clearly identify the trajectories of the implanted cannulas. All animals were confirmed to have successful bilateral cannula implanted into their desired region.
2.2.7 Data analyses

Data and statistical analyses for the study were performed using Excel and GraphPad Prism software. The output data from the operant chambers were initially extracted using the MedPC to Excel software. In Excel, custom formulas were employed to conduct preliminary analyses of this data. Subsequently, the data was imported into GraphPad Prism software, which facilitated more advanced statistical analyses and data visualization.

For the data obtained from the Morris Water Maze, the Pathfinder software package was utilized for analysis. This software is specifically designed to analyze spatial navigation tasks like the Morris Water Maze and provides detailed insights into the performance of the subjects in such tasks.

Elevated Plus Maze data was recorded with a Logitech webcam and analyzed manually for time spent in the open or closed arms. One animal with ventral hippocampal inactivation was excluded because the behavioural video was corrupted.

In terms of statistical evaluation, a combination of Analysis of Variance (ANOVA) statistical models and unpaired t-tests were employed to determine the significance of the findings. ANOVA is useful for comparing more than two groups, while t-tests are typically used for comparisons between two groups. This comprehensive approach ensured a robust and thorough analysis of the experimental data, allowing for accurate interpretation of the results and their significance (p < 0.05) in the context of the study's objectives.
2.3 RESULTS

To ascertain the potential role of the hippocampus in probabilistic reversal learning, a targeted inactivation technique was utilized. This approach involved the agonism of GABA\(_a\) and GABA\(_b\) receptors, which are inhibitory receptors prominently expressed on neurons within the hippocampus. By activating these receptors, the neuronal activity in the hippocampus was effectively suppressed. All probabilistic reversal task data are from within-subjects comparisons with infusion of either muscimol/baclofen or vehicle. Test data is shown with an N of 10 animals constituting 4 ventral (1F, 3M) and 6 dorsal (2F, 4M) rats.

2.3.1 Rats continue to improve in performance in the probabilistic reversal paradigm long-term

Throughout the 9-week training paradigm, the rats exhibited a progressive improvement in both reversal learning and correct lever selection behaviors. Notably, there was a discernible trend of increasing mean successful reversals, accompanied by a higher frequency of correct lever presses (Figure 7). During the last 2 weeks of training, males continued to show an increase in correct presses (linear regression, F\(_{(1, 86)}\) = 5.8, p = 0.02).

Figure 7. Training data over 45 days. A. Animals continue to make more reversals over time. B. Animals learn quickly to respond greater than 50% of the time on the correct lever and continue to make improvements throughout the training period.
2.3.2 Female rats respond at a significantly lower rate than male animals

In the context of probabilistic reversal learning, we observed that female rats exhibit a notable pattern of behavior, particularly in terms of trial omissions. The data indicates that female rats tend to omit nearly 50% of the trials throughout the training period (t-test, p<0.0001) (Figure 8). Despite this high rate of omissions, their performance in terms of correct lever selection and normalized reversals showed improvement, mirroring the trend observed in male rats. However, the average rate of omissions remained consistently high throughout the entirety of the training period.

This pattern suggests a potential sex-based difference in approach or strategy to the probabilistic reversal learning task. The high omission rate in female rats could be indicative of a more cautious or selective approach to the task, possibly influenced by differences in stress response, motivation, or decision-making processes between male and female rats.

Figure 8. A sex difference in the number of total lever presses can be seen throughout training. Females respond to fewer trials than males.
2.3.3 Inactivation produced no significant change in reversals completed or correct lever presses

Neither dorsal nor ventral hippocampal inactivation had any significant effects on reversal behaviour or correct lever presses. Reversals showed no inactivation effect when dorsal/ventral inactivation was combined (paired t-test, p=0.54). Correct presses showed no effect of inactivation on performance (paired t-test, p=0.15) (Figure 9).

Figure 9. A. There was no significant effect of dorsal or ventral hippocampal inactivation using muscimol/baclofen on reversals completed. B. Reversals with inactivation groups (dorsal/ventral) combined. C. Reversals completed by male rats with inactivation groups (dorsal/ventral) combined. D. Reversals completed by female rats with inactivation groups (dorsal/ventral) combined. E. No significant effect of dorsal or ventral hippocampal inactivation using muscimol/baclofen on correct presses was found. F. Correct presses with inactivation groups (dorsal/ventral) combined. G. Correct presses completed by male rats with inactivation groups (dorsal/ventral) combined. H. Correct presses completed by female rats with inactivation groups (dorsal/ventral) combined.
2.3.4 Hippocampal inactivation reduced Win-Stay behaviours

The silencing of both the dorsal and ventral regions of the hippocampus decreased 'win-stay' behavior during the probabilistic reversal learning task (Figure 10). In this context, 'Win-Stay' behavior refers to the tendency of an animal to repeat lever press that previously led to a reward. Win-stay behaviour and lose-shift behaviours were analyzed on both correct and incorrect levers with a significant decrease after inactivation independent of region (paired t-test, p=0.02). Lose-shift behaviour showed no effect of inactivation (2-way ANOVA; Sex F(1,16) = 1.2, p = 0.27; Inactivation F(1,16) = 0, p = 0.83).

![Graphs A to H showing win-stay and lose-shift behaviours](image)

Figure 10. Animals perseverate on a rewarded lever less under hippocampal inactivation. A. Win-stay behaviour in both dorsal and ventral inactivated animals. B. When dorsal and ventral groups are combined, inactivated animals show significantly lower win-stay percentages (t-test, p=0.023) C. Male animals drive the inactivation effect on win-stay behaviour. D. Female rats do not show a significant inactivation effect for win-stay behaviours. E. Lose-shift behaviour on both levers did not show any trends after inactivation. F. Lose-shift effects remain absent with groups combined. G & H. Male and Female lose-shift behaviours under vehicle and inactivation, respectively.
2.3.5 Hippocampal inactivation reduced response latency

The inactivation of both the dorsal and ventral regions of the hippocampus decreased response latency during the probabilistic reversal learning task (2-way ANOVA; Sex $F_{(1,16)} = 1.47$, $p = 0.24$; Inactivation $F_{(1,16)} = 6.29$, $p = 0.023$). No differences were noted in number of omissions (2-way ANOVA; Sex $F_{(1,16)} = 0.1$, $p = 0.71$; Inactivation $F_{(1,16)} = 3.4$, $p = 0.08$) (Figure 11).

2.3.6 Post-surgical changes in behaviour

During the post-surgical period, we see some changes in task-specific behaviours. In males, we see a reduction in lever presses during the post-surgical retraining and testing phases (t-test, $p = 0.02$). Females do not show the same effect of surgery (t-test, $p = 0.11$). Notably, the
direction of the change in means are opposite between male and female animals. We don’t see any effect of surgery on reversals completed (Figure 12).

![Figure 12. Plots of reversals (A) and lever presses (B) during testing phase, post-surgical retraining, and the final week of training. A. Reversals completed during the phases of training and testing in males and females. B. Lever presses during the training and testing periods. Males show a reduction in lever pressing during the post-surgical period (t-test, p=0.02).]

2.3.7 Behavioural controls

In our Morris Water Maze experiments, animals subjected to dorsal hippocampal inactivation (n=6) exhibited impaired navigational performance compared to their counterparts administered with the vehicle solution through ventral hippocampal cannula (n=4). Latency to find the escape platform was not significantly different between groups (t-test, p=0.36). Spatial vs non-spatial strategies were significantly different (t-test p = 0.0009). A strategy analysis using Pathfinder software revealed a marked increase in non-spatial behaviors, such as thigmotaxis, in the inactivated group relative to control animals (Figure 13). These findings align with those of Micheau et al., who reported similar behavioral alterations following dorsal hippocampal inactivation (Micheau et al., 2004).
Figure 13. Morris Water Maze swim strategy analysis shows an increase in non-spatial strategies, such as thigmotaxis in animals with dorsal hippocampal inactivation. A. Search path strategy distribution in vehicle-treated animals with ventral hippocampus cannula. B. Search strategy distribution in animals with dorsal hippocampal inactivation. C. Spatial (Direct Path through Semi-focal search) vs non-spatial (Scanning through thigmotaxis) strategy distribution in control or dorsal hippocampus inactivated animals.

In the Elevated Plus Maze (EPM) test, a trend was observed where animals with ventral hippocampal inactivation (n=3) demonstrated altered exploratory behavior. Quantitatively, these animals spent more time in the open arms (t-test, p = 0.12), and less in the closed arms, compared to controls (n=6) (t-test, p = 0.047) (Figure 14). These findings suggest an alteration in ‘anxiety-like’ behavior, consistent with the established role of the ventral hippocampus in modulating such responses (Forro et al., 2022).

Figure 14. Time spent in the open or closed arms of the Elevated Plus Maze. Animals that underwent ventral hippocampal inactivation spent more time in the open arms and less time in the closed arms of the maze (t-test, p=0.047).
2.4 Discussion

The training behavior of the animals in this experiment, conducted without pharmacological intervention, revealed significant findings. Notably, a marked sex difference in task behavior was observed, with female rats showing a higher rate of trial omission, or less lever presses, per session compared to males. Additionally, the experiment demonstrated the rats' capacity for continued improvement in the probabilistic reversal learning paradigm, even after extensive training.

The experiment further established that inactivation of both the dorsal and ventral regions of the hippocampus led to behavioral changes in the probabilistic reversal learning task. Specifically, the animals exhibited deficits in their ability to adapt their behavior based on past rewards. Furthermore, the rats responded more quickly during trials, which could suggest increased impulsivity following the inactivation of both the dorsal and ventral hippocampal regions. This is in contrast to evidence suggesting that acute stress may increase choice latency in probabilistic reversal learning (Bryce & Floresco, 2021). Interestingly, the experiment did not yield clear distinctions between the roles of the dorsal and ventral hippocampus in driving these behavioral changes. This is supported by the growing evidence that both dorsal and ventral hippocampus play roles in probabilistic and statistical behaviour. The dorsal hippocampus’ contribution to declarative episodic memory and updating the task representation over time is hypothesized to contribute to the deficits observed during this experiment. Alternatively, the ventral hippocampus’ contributions to reward processing, as well as its role in spatial mapping relative to reward, provide evidence of its role in these behavioural effects.

However, there were several limitations to the experiment. Firstly, a significant number of animals (six) had to be euthanized before behavioral testing due to signs of infection, raising
concerns about the potential impact of altered baseline behavior on test results. As a precaution, any animals showing signs of behavioural changes were not included in the testing. Secondly, the drug infusion process, involving muscimol and baclofen, was stressful for the rats. Despite habituation with mock infusions, there were differences between vehicle test data and training data, suggesting that the surgery or infusion process itself may have influenced the animals' behavior. These differences were noted immediately after surgery, and were not completely recovered after the post-surgical retraining period.

In light of these limitations, a decision was made to adopt a less invasive, chemogenetic approach in subsequent experiments. Chemogenetics offers a method to modulate neuronal activity with reduced stress and invasiveness compared to direct drug infusions, potentially providing more reliable insights into the hippocampal function in learning and decision-making processes. This shift in methodology aligns with recent advances in neuroscience research, emphasizing the use of minimally invasive techniques for studying brain function (Fenno et al., 2011; Roth, 2016). The shift to a chemogenetic DREADD approach was expected to reduce postsurgical recovery time and infection risk. Additionally, CNO injections are less distressing to the animals prior to behavioural tests when compared to drug infusions through cannula. This thereby minimizes the confound of stress on test results.
Chapter 3: Dorsal hippocampal inactivation using chemogenetics in Probabilistic Reversal Learning

3.1 Introduction

The exploration of the roles of hippocampal subregions in probabilistic reversal learning unveils a complex landscape of neural specialization within neuroscience research. Emerging evidence underscores a differential engagement of these subregions in the learning process, with each contributing uniquely to various aspects of behavior. This specificity highlights the intricate architecture of the hippocampus and its functional segmentation, underlining the necessity of nuanced approaches to understand its role in learning and memory.

To delve deeper into the hippocampus’ involvement in probabilistic learning, our study harnessed chemogenetic techniques. Chemogenetics has become an indispensable tool in behavioral neuroscience due to its unparalleled specificity and efficacy in modulating neuronal activity (Roth, 2016). Traditional pharmacological methods face limitations in achieving the desired precision and temporal control over neuronal function. In contrast, Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) represent a significant advancement by offering meticulous manipulation of neuronal activity. These tools allow for viral tagging of specific cell populations, and excitation or inhibition of these tagged cells with acute administration of a drug, CNO. This is especially valuable for investigating intricate neural networks such as the hippocampus, where precise activation or inhibition of neuronal populations is crucial (Urban & Roth, 2015).
The dorsal hippocampus, in particular, is renowned for its central role in cognitive functions and spatial memory, serving as a crucial hub for the encoding and retrieval of spatial information. This capability is vital for probabilistic reversal learning, wherein the ability to adapt to changing spatial contingencies is tested. Studies have illuminated the dorsal hippocampus’ contribution to animals' navigational skills, enabling them to reorient and recalibrate their strategies in response to new spatial arrangements. Such adaptability is foundational to successful engagement in probabilistic reversal learning tasks, demonstrating the dorsal hippocampus’ indispensable role in facilitating cognitive flexibility and spatial learning.

Furthermore, the engagement of the dorsal hippocampus in these learning processes is not merely passive. It involves active manipulation of spatial memories and representations. This suggests a dynamic interplay between the encoding of new spatial information and the retrieval of existing memories, allowing animals to make informed decisions in the face of uncertainty and change. This is especially important in the reversal aspect of our task. The animal must update its representation of the trial based on the feedback of past trials. The dorsal hippocampus’ ability to integrate and reorganize spatial information underscores its contribution to higher-order cognitive processes, such as decision-making and problem-solving, which are essential for navigating the complexities of probabilistic reversal learning.

The hippocampus has been shown to be critically involved in model-based decisions. Human fMRI research has implicated the hippocampus in both model-free and model-based learning, implying crosstalk with the striatum (Bornstein & Daw, 2013). Additionally, evidence from a human probabilistic reversal learning task demonstrated that patients with hippocampal lesions performed sub-optimally compared to hippocampally intact controls (Jocham et al., 2009;
Vilà-Balló et al., 2017). This evidence points to hippocampal involvement in tasks with similarities to our rodent model.

Probabilistic reversal learning tasks in rats are instrumental for probing the neural circuits and mechanisms underlying cognitive flexibility, decision-making under uncertainty, and adaptability to shifting environmental cues (Floresco, 2015).

In this research, we utilized an inhibitory DREADD to transiently inactivate the dorsal hippocampus, prompted by preliminary findings that underscored the necessity for a minimally invasive examination of hippocampal functions. Acute stress has been demonstrated to reduce motivation to respond in similar tasks (Bryce & Floresco, 2021). The dorsal hippocampus was targeted based on its pivotal role in cognitive operations, aiming to bolster the statistical robustness of our investigation by minimizing the variability potentially introduced by including multiple hippocampal subregions. This approach was further justified by our pharmacological inactivation data, which did not indicate significant functional disparities among dorsal or ventral hippocampal subregions.

The application of inhibitory DREADDs in our study was a deliberate strategy to refine our insight into the behavioral role of the dorsal hippocampus. In contrast to pharmacological inactivation, a chemogenetic approach is hypothesized to lead to lesser surgical recovery times, lower rates of post-surgical infection, and lower levels of pre-test stress.

3.2 Methods

3.2.1 Animals

Consistent with our first experiment, male and female Long Evans rats were utilized. Animals were again acclimated to handling by all experimenters to mitigate any stress associated
with handling during the experiments. Throughout the behavioural training and testing phases, the animals were subjected to food restriction.

In experiment 2, 16 Long-Evans (8 male, 8 female) rats were acquired from Charles-River Laboratories. Animals were habituated to their new environment for 1 week. All rats were initially trained to lever-press for a 40mg sucrose reward. Animals were then trained on the probabilistic reversal learning task. After 6 total weeks of training, all animals underwent AAV injection surgery into dorsal hippocampus. Animals were given 2 weeks to recover from the surgery and 2 more weeks to ensure maximal expression of the AAV DREADD virus. Animals were then re-trained on the PRL task prior to testing for 2 weeks. Following retraining, animals were injected with either vehicle or CNO, alternating every second day for 12 days (3 vehicle test sessions, 3 CNO test sessions per animal). For Morris Water Maze testing, 4 male and 4 female animals were randomly selected to receive CNO for the 3-day testing paradigm. The other 8 animals received vehicle injections for all 3 days. Animals were sacrificed immediately after the last MWM session.

Experiment 3 had 16 animals (8 males and 8 females) assigned to an AAV group which would receive surgery. Another 16 (8 male, 8 female) age-matched animals were included as non-surgical controls. Animals were habituated to their new environment for 1 week, after which the AAV animals underwent AAV injections bilaterally into dorsal hippocampus. Animals were allowed 2 weeks to recover from surgery. Control animals remained in home-cage during the surgical and recovery period. All rats were initially trained to lever-press for a 40mg sucrose reward. Animals were then trained on the probabilistic reversal learning task. After 5 total weeks of training, all animals began testing. Animals were injected with either vehicle or CNO, alternating every second day for 12 days (3 vehicle test sessions, 3 CNO test sessions per
For Morris Water Maze testing, all AAV animals received CNO for the 3-day testing paradigm. The non-surgical control animals received vehicle injections for all 3 days. Animals were sacrificed immediately after the last MWM session (Figure 15). All animal work was done in accordance with UBC’s animal care regulations.

**Figure 15.** Timeline comparison of the first DREADD experiment (Experiment 2) and the second DREADD experiment with non-surgical controls (Experiment 3). Non-surgical controls received identical treatment as AAV animals after 5 weeks.

### 3.2.2 Food restriction

Throughout the experiments, all rats were subjected to food restriction, maintaining their body weight at approximately 85% of their ad libitum (A.L.) weight. This weight management was closely monitored in accordance with a Charles River growth chart. During any surgical
intervention and the subsequent recovery phases, the animals were returned to ad libitum feeding to support their health and recovery (Figure 16).

Figure 16. Weights of the animals plotted over the experiment. Animals under food restriction stabilised early in the experiment and continued to gain weight. A. Weights of female animals in Experiment 2. B. Weights of male animals in Experiment 2. C. Weights of female animals in Experiment 3. D. Weights of male animals in Experiment 3

3.2.3 Probabilistic reversal learning task

The operant-chamber based probabilistic reversal task developed by Dr. Stan Floresco that was used in our previous experiments was utilized again for these tests. Reward
contingencies remained at 80% for the correct and 20% for the incorrect levers. Reversal criterion was set at 8 consecutive trials.

Each daily session comprised 200 trials for each animal. All training sessions were conducted once daily during the light phase of the light-dark cycle. Upon receiving a reward, a 40mg sucrose pellet was dispensed into the food hopper.

Behavioural data from these sessions were recorded and exported using Med Associates’ “MED-PC” and “MED-PC to Excel” software, respectively. This data collection method ensured accurate and comprehensive recording of the rats' performance throughout the task.

3.2.4 Chemogenetics

In an effort to minimize stress on the animals and significantly reduce the risk of infection compared to cannulated animals, chemogenetics were employed. The chosen vector was adeno-associated virus serotype 2 (AAV2). This vector contained the synapsin promoter (hSyn) and an inhibitory hM4D(Gi) with mCherry. A volume of 1μl of AAV2-hSyn-hM4D(Gi)-mCherry (with a titer of 5.00E+12 GC/ml) was infused into each hemisphere of the dorsal hippocampus at coordinates 3.6mm posterior, 2.2mm lateral relative to bregma, and 2.5mm ventral to dura. The infusion was conducted over a 5-minute duration at a rate of 0.2μl/minute. To ensure effective viral delivery, the syringe remained in place for an additional 5 minutes after the injection before being gradually retracted, thus minimizing potential backflow of the virus.

All surgeries were performed under isoflurane anesthesia, with animals receiving meloxicam and bupivacaine for analgesia.

Animals underwent testing 6 weeks following surgery to allow for viral expression. In experiment 2, the first chemogenetic experiment, a 4-week period was allowed for viral
expression before the animals were retrained to criterion and tested. Experiment 3 was designed to expedite the process by training the animals after they underwent the AAV injection surgery (see experimental timeline, Figure 15). Thirty minutes before testing, the animals were administered either 3 mg/kg clozapine N-oxide (2 mg/ml CNO, dissolved in a 5% Dimethyl Sulfoxide (DMSO) in 0.9% Saline solution) or a vehicle solution (5% Dimethyl Sulfoxide in 0.9% Saline solution). This protocol was designed to optimize the conditions for evaluating the effects of chemogenetic intervention on the rats' behaviour in the context of the experiments.

3.2.5 Non-surgical controls

To robustly validate our experimental findings, Experiment 3 included a non-surgical control group. This control is critical for differentiating the effects of chemogenetic manipulation from other potential variables. The control group was administered intraperitoneal (IP) injections of either clozapine-N-oxide (CNO) or a vehicle solution.

The inclusion of CNO in the control group is pivotal. CNO is the ligand used to activate DREADDs. Using CNO in control animals tests for any intrinsic pharmacological effects independent of DREADD activation. This approach aligns with the recommendations of Gomez et al., who emphasize the importance of verifying that observed effects in DREADD experiments are truly due to receptor activation rather than non-specific actions of CNO (Gomez et al., 2017). Similarly, the vehicle solution serves as an additional control to account for any effects arising from the injection procedure itself.

These controls ensure that the behavioural and physiological changes observed in the experimental groups can be confidently attributed to the specific activation or inhibition of the
targeted neural circuits by DREADDs, rather than to the injection process or the systemic effects of CNO.

3.2.6 Morris Water Maze Control

Similar to previous experiments, to confirm the behavioural effects of hippocampal inactivation, animals were subjected the Morris Water Maze (MWM), a well-established test for evaluating spatial navigation, a task that is predominantly dependent on the functionality of the dorsal hippocampus. In the MWM, each animal was required to locate a hidden, submerged platform in a 2-meter diameter pool filled with water maintained at 22°C. The water was rendered opaque with non-toxic paint to obscure the platform. Animals were given 60 seconds per trial to find the platform, with the starting position varied across trials to prevent the formation of a simple route-following strategy. If an animal failed to locate the platform within this timeframe, it was gently guided to the platform by the experimenter. This step was crucial for facilitating spatial learning and memory formation. Data collected during these trials, including escape latency and path efficiency, were analyzed to determine the extent of spatial learning impairments due to hippocampal inactivation. Animals were trained for 3 days, 8 trials per day.

3.2.7 Tissue Collection

Following euthanasia, animals underwent perfusion with paraformaldehyde (PFA), a standard fixative in histological procedures due to its ability to preserve tissue morphology (McLean & Nakane, 1974). This was immediately followed by the extraction of the brains, which were then immersed in a 4% PFA solution for further fixation. The 4% concentration is
commonly used to ensure adequate tissue preservation while minimizing potential tissue damage (Kiernan, 2000).

For tissue sampling and analysis, the brains were sectioned using a vibratome. This method is preferred for its precision in cutting uniform slices without significant distortion of the tissue structure. The brain slices were prepared at a thickness of 55μm. This thickness was strategically chosen to optimize the balance between structural integrity and the effective diffusion of staining or labeling agents. Such a thickness also facilitates the precise localization of labeling, which is crucial for accurate morphological analysis.

In our analysis, we ensured that only data from samples with successful infusions into the dorsal hippocampal region were included. This selective criterion is critical to maintain the validity and reliability of our findings, as it ensures that the observed effects are indeed attributable to the targeted brain region.

3.2.8 Histology

To verify the adequacy of the injection volume, viral infection, and spread of the virus, the brain tissue slices were subjected to staining procedures targeting specific markers. The slices were stained for red fluorescent protein (RFP), which binds to the mCherry reporter present in the AAV2-hSyn-hM4D(Gi)-mCherry virus used in the experiments. Additionally, DAPI (4′,6-diamidino-2-phenylindole) staining was employed to highlight the nuclei of neurons in the tissue (Figure 17).

Once stained, the tissues were mounted for examination and analyzed using a Leica confocal microscope. This detailed analysis involved identifying sections that demonstrated fluorescence, indicative of successful viral infection and spread.
This approach ensured that histological data from animals could be compared to behavioural outcomes in order to ensure any conclusions drawn were warranted, thereby maintaining the integrity and accuracy of the experimental findings.

![Representative RFP expression in dorsal hippocampus after DREADD injections.](image)

*Figure 17. Representative RFP expression in dorsal hippocampus after DREADD injections.*

### 3.2.9 Data analyses

Data and statistical analyses for this study were conducted using a combination of Excel, GraphPad Prism, and Pathfinder software. Preliminary analyses, including basic computations and data organization, were initially performed in Excel. This data was then imported into GraphPad Prism, which facilitated advanced statistical analyses and enhanced data visualization capabilities. For the Morris Water Maze data, the Pathfinder software package was specifically
utilized due to its tailored design for analyzing spatial navigation tasks. This software provided
detailed insights into the subjects' performance, enabling a deeper understanding of their
navigational strategies and efficiencies.

Regarding statistical evaluation, our approach employed a combination of Analysis of
Variance (ANOVA) models and t-tests. ANOVAs were utilized where comparisons across groups
with more than one factor of interest were needed. Sex, CNO/Vehicle, and AAV/Control were
compared. Post-hoc analyses were conducted where necessary to determine the significance of
effects. Linear regression was employed when looking for trends in the viral expression levels of
animals.

3.3 Results

3.3.1 Experiment 2

After histological verification, 3 female animals and 1 male animal were excluded from
analysis. Data are presented with an n of 12. In our second experiment, we successfully
replicated the sex differences observed in our previous work during training phases. Specifically,
female animals exhibited a reduced response rate compared to their male counterparts (2-way
ANOVA; Sex $F(1,14) = 18.28, p = 0.0008$, Day $F(7.8, 109.2) = 8.0, p < 0.0001$) (Figure 18).

![Figure 18. Female rats make more omissions than male rats during training.](image-url)
Reversal behaviours, where the animal responds 8 times consecutively on the correct lever and the contingencies switch, differed between sexes (2-way ANOVA; Sex $F_{(1,20)} = 8.0$, $p = 0.01$; CNO $F_{(1,20)} = 3.2$, $p = 0.08$). Post-hoc analyses revealed the effect of CNO in female animals did not reach statistical significance (paired t-test, $p = 0.06$) (Figure 19).

![Graphs showing reversals and correct presses for vehicle and CNO treated groups for males and females.](image)

*Figure 19. A-C. Reversal performance in rats treated with vehicle or inactivated with CNO. Female inactivation $p=0.06$. D-F. Correct presses performed by animals under vehicle or CNO induced inactivation.*

However, no significant differences were observed between the control and hippocampal inactivation groups in win-stay behaviour (paired t-test, $p = 0.34$). No sex-specific effects were shown (paired t-test; males $p = 0.69$, females $p = 0.44$). Lose-shift behaviour was not affected by inactivation through DREADDs. Lose-shift was not significantly different between groups.
(paired t-test, p = 0.09). There were also no inactivation effects when male or female animals were examined (paired t-test; males p = 0.38, females p = 0.19) (Figure 20).

Figure 20. A-C. We saw no effect on perseverative Win-Stay behaviour. D-F. We also see no significant inactivation effects on lose-shift behaviours.

Response latency did not change following inactivation of the dorsal hippocampus with CNO (paired t-test, p = 0.86). Omissions showed a significant sex effect (2-way ANOVA; Sex $F_{(1,20)} = 13.58$, p = 0.0015; CNO $F_{(1,20)} = 0.004$, p = 0.94). There was no effect of CNO inactivation (paired t-test, p = 0.52) (Figure 21).
To verify the efficacy of our chemogenetic dorsal hippocampal inactivation, rats were behaviourally trained and tested in the Morris Water Maze (MWM), a paradigm widely recognized for evaluating spatial navigation abilities, a key function of the dorsal hippocampus. Given the well-documented role of the dorsal hippocampus in spatial memory and navigation, we anticipated reduced performance by the inactivated group. A strategy analysis using Pathfinder software revealed an increase in non-spatial behaviours in the inactivated group relative to control animals during the first day of testing, but the difference was not significant (Vehicle $\mu = 8$, $\sigma = 2$, CNO $\mu = 6.5$, $\sigma = 1$; $p = 0.13$) (Figure 22). There were no significant
group differences in escape latency between CNO and vehicle treated animals (one-way ANOVA; F_{(3, 8)} = 0.26, p = 0.85).

**Figure 22.** Morris Water Maze search strategy analysis. **A.** Strategy distribution for the 3-day water maze task. Animals with inactivation showed elevated random search and thigmotaxis compared to vehicle. **B.** The first day of the water maze test. Vehicle animals show more spatial strategies (μ = 8) compared to those with dorsal hippocampal inactivation (μ = 6.5) but the difference was not significant (t-test, p=0.13).

### 3.3.2 Experiment 3

To corroborate the findings of our second experiment, we replicated the study using another cohort of Long Evans rats, both male and female. Additionally, a non-surgical control group of 16 animals was included to provide a baseline comparison. Unlike our previous
DREADD experiment (*Experiment 2*), animals in Experiment 3 underwent surgery prior to any training. This was done to allow the DREADD virus to express during the training period, avoiding a lengthy post-surgical rest period (timeline, Figure 15). Otherwise, the methodology was kept consistent.

Female animals again exhibited a reduced response rate compared to their male counterparts (3-way ANOVA; Sex $F_{(1, 126)} = 21.15$, $p < 0.0001$, AAV $F_{(1, 126)} = 5.3$, $p = 0.02$, Day $F_{(17, 126)} = 34.83$, $p < 0.0001$) (Figure 23).

![Figure 23. Male and female animals make significantly different number of omissions during the training period.](image)

Interestingly, our results revealed significant behavioural differences between the virally injected animals (AAV) and the non-surgical controls. Notably, control animals exhibited a significantly higher number of reversals (3-way ANOVA; AAV $F_{(1, 14)} = 8.2$, $p = 0.01$; Sex $F_{(1, 14)} = 0$, $p = 0.89$; CNO $F_{(1, 14)} = 0$, $p=0.93$), with this effect being predominantly observed in male subjects (paired t-test, $p = 0.02$). There were also significant differences in the number of correct presses during the task (3-way ANOVA; AAV $F_{(1, 14)} = 10.47$, $p = 0.006$; Sex $F_{(1, 14)} = 2.5$, $p =$
0.14; CNO $F_{(1, 14)} = 1.5, p=0.23$). Post-hoc analyses revealed significant contributions of both sexes (2-way ANOVAs; Male: AAV $F_{(1, 28)} = 10.9, p = 0.003$, CNO $F_{(1, 28)} = 1.0, p = 0.31$, Female: AAV $F_{(1, 28)} = 6.3, p = 0.02$, CNO $F_{(1, 28)} = 0.7, p = 0.4$) (Figure 24).

![Figure 24](image)

**Figure 24.** A-C. Reversal behaviour under Vehicle or CNO injections in the Control or AAV groups. D-F. Correct presses completed during the probabilistic reversal task under Vehicle or CNO injections in the Control or AAV groups.

Additionally, control animals displayed more frequent win-stay behaviours. There were no significant effects of sex or CNO administration (3-way ANOVA; AAV $F_{(1, 56)} = 13.47, p = 0.0005$; Sex $F_{(1, 56)} = 0.01, p = 0.90$; CNO $F_{(1, 56)} = 0.14, p = 0.71$). Post-hoc analyses showed the effect was only significant in the male animals (2-way ANOVA; AAV $F_{(1, 28)} = 10.51, p = 0.003$, CNO $F_{(1, 28)} = 0.49, p = 0.49$). Lose-shift behaviour during test trials a significant sex effect (3-
way ANOVA; AAV $F_{(1,56)} = 2.7, p = 0.10$; Sex $F_{(1, 56)} = 31.3, p < 0.0001$; CNO $F_{(1,56)} = 0.50, p = 0.48$). Post-hoc analysis revealed an effect of AAV surgery in females on the incorrect lever (2-way ANOVA; AAV $F_{(1,28)} = 4.4, p=0.046$; CNO $F_{(1,28)} = 0.02, p=0.89$) (Figure 25).

![Figure 25](image)

*Figure 25. A-C. Win-Stay behaviours under Vehicle or CNO injections in the Control or AAV groups. D-F. Lose-Shift behaviours task under Vehicle or CNO injections in the Control or AAV groups.*

Response latency did not show an effect of inactivation or a difference between AAV and Control groups. (Repeated measures 2-way ANOVA; AAV $F_{(1,30)} = 0.59, p = 0.45$; CNO $F_{(1,30)} = 1.24, p = 0.27$). However, there was a strong sex-dependant interaction of AAV on omissions (3-way ANOVA; AAV $F_{(1,56)} = 0.21, p = 0.64$; Sex $F_{(1,56)} = 60.1, p < 0.0001$; CNO $F_{(1,56)} = 0.003, p = 0.95$. AAV x Sex Interaction: $F_{(1,56)} = 11.9, p = 0.001$). Post-hoc analyses revealed different direction effects in male and female animals on omissions (2-way ANOVA; Males: AAV $F_{(1,28)} =$
7.5, p = 0.01; CNO $F_{(1,28)} = 0.15, p = 0.71$, Females: AAV $F_{(1,28)} = 5.4, p = 0.03$; CNO $F_{(1,28)} = 0.10, p = 0.75$) (Figure 26).

Figure 26. A-C. No difference in response latency are reported between control and inactivated animals and under CNO or Vehicle injections. D. There is no global effect of omissions, however, there is a Sex x AAV interaction effect. E. Male animals omit more after AAV surgery relative to controls (p = 0.01). F. Female animals omit less after AAV surgery when compared to controls (p = 0.03).

To verify the efficacy of our chemogenetic dorsal hippocampal inactivation, rats were behaviourally trained and tested in the Morris Water Maze (MWM), a paradigm widely recognized for evaluating spatial navigation abilities, a key function of the dorsal hippocampus. Given the well-documented role of the dorsal hippocampus in spatial memory and navigation, we anticipated reduced performance by the inactivated group. Latency to reach the escape platform and swim-path length were not different between vehicle and CNO injected groups. A strategy analysis using Pathfinder software failed to reveal any group differences (Figure 27).
Figure 27. Morris Water Maze search strategy analysis. A. Strategy distribution for the 3-day water maze task. We did not see any measurable differences between groups. B. The first day of the water maze test. There are no pronounced differences between inactivated and vehicle animals.

Since we were unable to demonstrate group differences in the Morris Water Maze, we calculated the area of expression of the DREADD virus. Only five out of sixteen animals showed significant expression of mCherry. We did not show any correlations between level of expression of the DREADD and performance in the probabilistic reversal learning paradigm using linear regression analyses (Figure 28).
Figure 28. Plots of key behaviours vs. area of expression. Performance is from CNO test trials. A. Correct presses in the probabilistic reversal learning plotted against area of DREADD expression in the hippocampus. B. Reversals plotted against area of DREADD expression in the hippocampus. C. Win-stay behaviour plotted against area of DREADD expression. D. Latency to respond in the probabilistic reversal learning plotted against area of DREADD expression. E. Omitted trials plotted against area of DREADD expression in the hippocampus. F. Plot of z-score for key behaviours plotted against area of expression.
3.4 Discussion

This research offers new perspectives on the dorsal hippocampus’ role in probabilistic reversal learning, challenging some of our initial observations. Specifically, in experiment 2, our attempts to replicate the patterns noted in our first pharmacological inactivation experiment did not yield consistent group differences in metrics such as win-stay behaviors, reversal learning, and response times.

We found a significant difference in omissions between male and female rats. This is consistent with previous evidence that female animals respond less during the probabilistic reversal task (Bryce &Floresco, 2021). Win-stay behaviours, which may relate to the ability to utilize recent experience to guide future decisions, did not differ between vehicle and CNO inactivated trials. Additionally, we saw no change in latency to respond after CNO inactivation of the dorsal hippocampal region. The ability for the animals to flexibly update task representation and reverse between the now incorrect and newly correct levers was also not affected by CNO inactivation. This is consistent with results in dorsal hippocampus inactivated animals in our pharmacological inactivation experiment. When looking at lose-shifts during the task, a proxy of negative feedback sensitivity, we saw no significant differences between intact and dorsal hippocampus inactivated animals.

There was a close-to-significant effect of inactivation in the female reversal group. This observation aligns with established research demonstrating sex-specific neural responses to hippocampal manipulations (Yagi & Galea, 2019). However, the effect was not significant. The differential impact of hippocampal inactivation on reversal behaviours in females might indicate underlying sex-based neurobiological differences, warranting further investigation.
In an effort to substantiate these outcomes, Experiment 3 was executed alongside a comparable group of animals that did not undergo AAV infusion surgery. Again, our results did not demonstrate any significant impact of clozapine N-oxide (CNO) administration on win-stay behaviors, the number of reversals, or response latencies. A pivotal observation was the influence of adeno-associated virus (AAV) injections on enduring changes in behavior, hinting at a crucial effect of this procedure on behavioral modulation. I propose that these effects may be linked to widespread inflammatory alterations influencing hippocampal activity, a notion supported by existing research on how inflammation can modify neural functions (Badimon et al., 2020).

Intriguingly, the specific deactivation of the dorsal hippocampus via CNO did not lead to additional notable changes in behavior, prompting further inquiry into the underlying mechanisms.

Reversal behaviors were significantly reduced in animals that receive AAV injection surgery. The effect was primarily driven by male animals. These findings were mimicked with win-stay behaviours; surgical animals performed fewer win-stays compared to non-surgical controls. Again, these group differences were more significant among male rats. Reversals and win-stay behaviours are reliant on the flexible updating of task structure and responding to previous trial rewards. Deficits in these behaviours could be explained by decreased hippocampal processing (Bornstein et al., 2017; Bornstein & Daw, 2013; Vikbladh et al., 2019). We also observed an effect in the surgical group with respect to lose-shift behaviour, specifically on the incorrect lever. The effect was seen in female animals. Females who were surgically infused with AAV were more likely to change levers following no reward.

Moreover, the potential for surgical procedures themselves to elicit lasting behavioral modifications should not be overlooked and merits additional exploration. Our study did not find
a relationship between the degree of viral expression and behavioral outcomes. Furthermore, reproduction of the inactivation effects in experiment 2 did not support a female specific reversal difference. These results illuminate the complex relationship between hippocampal function and cognitive behavior, emphasizing the importance of experimental design and surgical intervention in behavioral neuroscience.
Chapter 4: Conclusion

4.1 Summary of results

The research spanned three experiments, each contributing unique insights into the role of the hippocampus in probabilistic reversal learning, with a particular focus on behavioral modifications following various interventions.

Experiment 1 highlighted significant behavioral findings in non-pharmacologically inactivated training trials, uncovering a sex difference in task performance, with female rats exhibiting a higher rate of trial omission. It showed that rats could improve in the probabilistic reversal learning task over long time periods. Pharmacologically inactivating both the dorsal and ventral hippocampal regions altered behavior, impacting the animals' ability to adjust actions based on past rewards and increasing impulsivity. The distinct contributions of the dorsal versus ventral regions remained unclear. Challenges included the euthanization of six animals due to infection risks and the stressful impact of drug infusion procedures on the rats, potentially affecting behavior.

Experiments 2 and 3 sought to replicate and extend the findings of Experiment 1, focusing on the effects of chemogenetic inactivation using clozapine N-oxide (CNO) and exploring the long-term behavioral impacts of adeno-associated virus (AAV) injections. Contrary to initial observations, no significant group differences were found in win-stay behaviors, reversal learning, and response times in Experiment 2. Similarly, Experiment 3, which included a control group of non-surgical animals, failed to demonstrate significant effects of CNO on the measured behaviors. However, it suggested that AAV injections might lead to behavioral changes potentially related to inflammatory alterations in hippocampal function, a hypothesis supported
by literature on inflammation's impact on neural dynamics. The experiments did not conclusively link viral expression levels to behavioral outcomes and failed to replicate possible sex differences in reversal behaviors observed in Experiment 2.

Although our findings could not be replicated in our follow-up experiments, the variability observed across the experiments serves as a testament to the hippocampus’ complexity, suggesting that its involvement in cognitive processes is far from homogeneous and highly dependent on the specific context of the intervention. Furthermore, the impact of AAV surgery on performance underscores the importance of including controls wherever possible. This complexity necessitates a meticulous approach to experimental design, where every variable, from the choice of intervention to the method of its application, is carefully calibrated to ensure the integrity and validity of the findings.

The impact of these interventions, as well as genetic manipulations like viral injections, on animal behavior illuminates the delicate balance within neural circuits that govern behavior. These interventions, while invaluable for dissecting the functional contributions of neural structures, also introduce variables that can profoundly alter the baseline behavior of the subjects. For instance, the stress associated with surgical procedures or the inflammatory responses elicited by viral vectors may themselves modulate neural activity and, by extension, behavior. Alternately, evidence suggest anesthetics such as the isoflurane used in these experiments can have impacts on hippocampal function (Uchimoto et al., 2014). Such effects underscore the necessity for control groups, such as non-surgical or sham-operated animals, to distinguish between the effects of the intervention and those of the procedure itself.
Furthermore, the unexpected findings—such as the lack of significant effects of clozapine N-oxide (CNO) on behavior in the face of substantial alterations induced by adeno-associated virus (AAV) injections—highlight the importance of considering long-term and systemic impacts of experimental interventions. These observations suggest that the behavioral effects of interventions may not be immediate or directly related to the intended manipulation of neural activity. They could arise from broader physiological changes, such as inflammation affecting neural dynamics across the brain. Overall, our work highlights the complexity of hippocampal involvement in cognitive processes and the importance of considering methodological nuances in behavioural neuroscience research.

In conclusion, the experiments collectively call for a heightened awareness of the complexities involved in studying the hippocampus’ role in cognition. They advocate for the adoption of rigorous experimental designs, the thoughtful consideration of all possible variables, and a cautious interpretation of results. Such diligence is essential for advancing our understanding of the hippocampal contributions to cognitive processes and for ensuring the reliability and reproducibility of findings in the field of behavioral neuroscience.

### 4.2 Application to the broader field

These data present a complex picture of hippocampal involvement in probabilistic reversal behaviours, as evidenced by the mixed results obtained from various experimental approaches. The findings also highlight the broader implications of changes in reward processing (i.e. changes in perseverative behaviours after reward) which can profoundly impact overall wellbeing. The ability to integrate past experiences and drive further action plays a major role in activities of daily living and is crucial to our effective functioning. Reward processing has been
shown to be impaired in disorders such as depression (Mueller et al., 2015). Understanding hippocampal contributions to reward processing may provide targets for further pharmacological development and therapeutic targets.

Furthermore, our observations that interventions such as AAV2 injections or surgical procedures may induce long-term behavioural alterations underscore the importance of considering the potential impact of these methods in neuroscience research. Of particular note is the possibility that AAVs, traditionally thought to be minimally inflammatory, could elicit inflammatory responses within the hippocampal structure. If confirmed, this would represent a critical insight into the use of AAVs in both research and clinical settings, as it challenges existing assumptions about their safety profile. This is supported by literature indicating that even minor inflammatory responses in the brain can have significant behavioural consequences (Won & Kim, 2020). Therefore, these findings not only contribute to our understanding of hippocampal functions in cognitive processes, but also highlight the need for rigorous evaluation of the long-term effects of experimental interventions in behavioural neuroscience.

4.3 Limitations

The inability to replicate the results obtained from GABA$_{a,b}$ agonist experiments using DREADDs could be attributed to several factors. Notably, DREADDs are often less effective in achieving the same level of neuronal inactivation as direct pharmacological methods. This difference in efficacy might be due to the distinct mechanisms through which DREADDs and GABA agonists modulate neuronal activity. Furthermore, recent research, including the work of Johnston et al. (2021), highlights that our selected AAV serotype, AAV2, might significantly impair neurogenesis in the dentate gyrus (DG) (Johnston et al., 2021). This is a critical
consideration, as the disruption of neurogenesis could profoundly affect hippocampal functions and, consequently, behaviours associated with reversal learning (Seib et al., 2020). These findings not only underscore the importance of methodological choice in neuroscience research, but also raise concerns about the potential side effects of certain experimental interventions. The impact of AAV2 on neurogenesis provides a pivotal insight into the need for careful evaluation of the long-term consequences of using specific AAV serotypes in brain research.

### 4.4 Future Directions

Future investigations in the realm of hippocampal function and its influence on behavior should allocate significant emphasis on demystifying the factors underlying the behavioral discrepancies observed between the control and AAV-injected groups noted in our third experiment. Achieving this necessitates a rigorous examination and refinement of the methodological approaches employed, ensuring that the effects attributed to AAV intervention are precisely isolated from other potential influences. This step is pivotal for affirmatively associating the observed outcomes with the inactivation of specific hippocampal regions through AAV-mediated gene delivery.

Upon establishing a robust link between AAV intervention and targeted brain region inactivation, research efforts should pivot towards a detailed analysis of the ventral hippocampus. Preliminary data indicate a substantial role of the ventral hippocampus in the behaviors under investigation, thus meriting its thorough examination in future studies. Our initial focus on the dorsal hippocampus was strategically chosen to maximize the statistical robustness of our findings. However, accumulating evidence underscores the necessity to explore the ventral hippocampus more closely, given its purported significant contributions to emotional
processing and stress responses, which could influence the behaviors assessed in our experiments (Bannerman et al., 2003; Fanselow & Dong, 2010). The functional dichotomy between the dorsal and ventral hippocampus, with the dorsal aspect primarily implicated in spatial navigation and memory and the ventral region in affective processes, provides a compelling rationale for this expanded investigative scope (Moser & Moser, 1998; Strange et al., 2014).

To further substantiate the distinct roles of these hippocampal subregions, it is imperative that future research employs a combination of advanced neuroscientific techniques. These may include optogenetics for precise activation or inhibition of specific neural circuits, high-resolution neuroimaging to observe functional changes in vivo, and behavioral assays tailored to dissect the contributions of each hippocampal subregion to various cognitive and emotional behaviors (Deisseroth, 2011; Liu et al., 2012). Such multi-faceted approaches will enable a more comprehensive understanding of the differential roles of hippocampal subregions, paving the way for nuanced insights into their contributions to complex behaviors.
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