Retroactive Interference of an Operant Discrimination induced by exposure to a Pavlovian-Conditioning Task: Task specific and neuronal factors

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Abstract

Retroactive interference (RI) is a phenomenon where a new learning experience interferes with the recollection of a previous one. This phenomenon has been mainly studied in rodents using behaviourally conflicting contextual memories and these forms of memories critically involve the normal functioning of the hippocampus. Studies have shown that silencing the activity of the hippocampus during the second (interfering) learning experience significantly impacts the strength of RI in a contextual memory paradigm.

The current study used the protocol established for the contextual memory paradigm and applied it to an appetitive learning setting that does not involve the hippocampus. Rats were first subjected to the learning of an action-outcome (A-O) association where pressing one of two levers yields a food reward. One hour later, rats were subjected to learning stimulus-outcome (S-O) association where rats are provided with cue that reliably predicts the delivery of food. The rats were tested on the A-O condition without reinforcement 24 hours later. Rats performed poorly in the A-O test when compared to a group that did not undergo the S-O condition. This suggests that the non-behaviourally conflicting S-O learning experience had interfered with the A-O task. These rats were also poorer in performance when compared to those who were tested immediately after experiencing the S-O association, suggesting that the S-O learning experience did not affect short term memory of the A-O task. Furthermore, when the delay between the initial A-O task and the rats were interfering S-O task was increased, the strength of RI tended to be diminished.

Based on these initial results, it was hypothesised that the common, critical neuronal substrate, the NAc core was also critical in the manifestation of retroactive interference in our paradigm. Therefore rats were subjected to neuronal silencing of the NAc core using the designer receptor exclusively activated by designer drug (DREADD) when undergoing the

classical conditioning paradigm. NAc inhibition did not impact the strength of RI. The results indicate that rats may have overlearned the initial task, and therefore future optimization of the protocol may increase the chances of obtaining a significant effect of NAc inhibition on RI.

This paradigm would also be useful in testing the validity of two conflicting theories of forgetting, the consolidation and the temporal distinction theories. The former suggests that the effect of RI is due to the prevention of memory storage and the latter suggests that it is due to confusion of the two stored memories during retrieval. The behavioural findings have in this study so far have agreed with both theories and a further manipulation is proposed to tease these methods of RI apart.

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Table of Contents

Abstract ..... **ii**

Acknowledgements ..... **iv**

Table of Contents ..... **v**

1 Introduction **1**

1.1 A Brief History of Retroactive Interference (RI) 2

1.2 Memory Disruptions in Patients with Neurological Damage 5

1.3 Retroactive Interference in Rodents 6

1.4 Temporary Neural Disruption in the Hippocampus 9

1.5 A-O and S-O Associations and the Nucleus Accumbens (Nac) 15

1.6 Current study 22

2 Methods **25**

2.1 Equipment 25

2.2 Subjects 25

2.3 Behavioural Protocol 26

2.3.1 Pellet Exposure 26

2.4 Preliminary Behavioural Training 26

2.4.1 Pellet training 26

2.4.2 Lever press training 26

2.5 Experiment 1 27

2.5.1 Behavioural conditions. 27

2.6 Experiment 2 29

2.7 Procedure 30

2.7.1 Surgery 30

2.7.2 Behavioural protocol 31

2.7.3 Histology 33

2.8 Data analysis 34

2.8.1 Experiment 1 34

2.8.2 Experiment 2 35

3 Results **36**

3.1 Experiment 1 36

3.1.1 T1 analysis 36

3.1.2 T2 analysis 37

3.1.3 T1-P analysis 37

3.2 Experiment 2 40

3.2.1 T1 analysis 40

3.2.2 T2 analysis 41

3.2.3 T1-P analysis 42

4 Discussion **44**

4.1 Experiment 1 45

4.1.1 Group 1 vs Group 2 45

4.1.2 Group 1 vs Group 3 46

4.1.3 Group 1 vs Group 4 47
4.2 Experiment 2 47
4.3 General discussion 50
4.4 Final remarks and implications 53
References 55

1 Introduction

Understanding how biological systems function normally can aid in understanding their pathologies. Scientists have therefore studied a non-pathological memory disruption such as retroactive interference (RI) to shed light on how memory is disrupted in disorders such as Alzheimer's disease and dementia. RI is a phenomenon that often occurs when two new learning experiences occur in serial order, and learning of the second task has a detrimental effect in the recollection of the first task at a later time. The second task is often referred to as an interfering, interpolated task for this reason (Dewar, Cowan, & Della Sala, 2007; Lechner, Squire, & Byrne, 1999; Wixted, 2004).

RI is often studied in relation to spatial learning, object location, and context dependant memories in rodents. Learning these tasks is highly reliant on the normal functioning of the hippocampus. By modulating the normal activity of the hippocampus during the learning of the interpolated task, scientists have been able to both alleviate and exacerbate the effect of RI in animals. Using the knowledge of RI in the context of hippocampal studies, the present study aimed to first investigate whether RI can be used to study memory related to appetitive motivation that does not involve the hippocampus (Corbit & Balleine, 2000), and further test whether, modulating the brain activity that is involved in these tasks can alleviate the effect of RI.

There are two common ways in which appetitive learning is measured: instrumental learning and Pavlovian learning. Appetitive instrumental learning is the gradual association of a particular behaviour (e.g., lever pressing in an operant chamber) with the delivery of food rewards and Pavlovian learning involves the association of a reliable environmental cue that predicts the delivery of food (Delamater, 2012). While the former is involved in the

association of an individual's action with an outcome (A-O) (Balleine, Delgado, & Hikosaka, 2007), the latter is the association of a stimulus with an outcome (S-O) (Gilroy, Everett, & Delamater, 2014), and both A-O and S-O learning involve intersecting brain regions like the nucleus accumbens (NAc) (Balleine & Killkross, 1994; Blaiss & Janak, 2009; Hernandez, Sadeghian, & Kelley, 2002; Kelley, Smith-Roe, & Holahan, 1997; Parkinson, Olmstead, Burns, Robbins, & Everitt, 1999). The current study therefore subjected rats to an A-O and S-O task in serial order and tested the performance of the rats in the A-O task in a probe session at a later time. Once sufficient evidence of retroactive interference was found, the current study further explored whether modulating neural firing in the NAc during the learning of the second task would have an effect in alleviating the RI seen in the first experiment.

1.1 A Brief History of Retroactive Interference (RI)

The term retroactive interference was first coined by Müller and Pilzecker (1900) while studying the effects of cue associated learning in humans. A list of paired nonsense-words were first taught (list A) where one of the two words acted as a cue and the other was intended to be the item of retention. Participants from both the control and experimental groups were presented with the cue at a later time and asked to verbally recite the associated word. The experimental and control conditions only differed in whether there was an interfering (interpolated) learning experience between the initial pair learning experience and the recall period. Participants from the experimental condition were required to learn a second list of paired nonsense-words (List X). During recall, participants made more errors in the experimental condition than those in the control group. The cognitive Neuroscience/Psychology literature credit this as the first study to empirically show the effect of RI (Dewar et al., 2007; Lechner et al., 1999; Wixted, 2004). Figure 1 provides a schematic representation of this experiment.

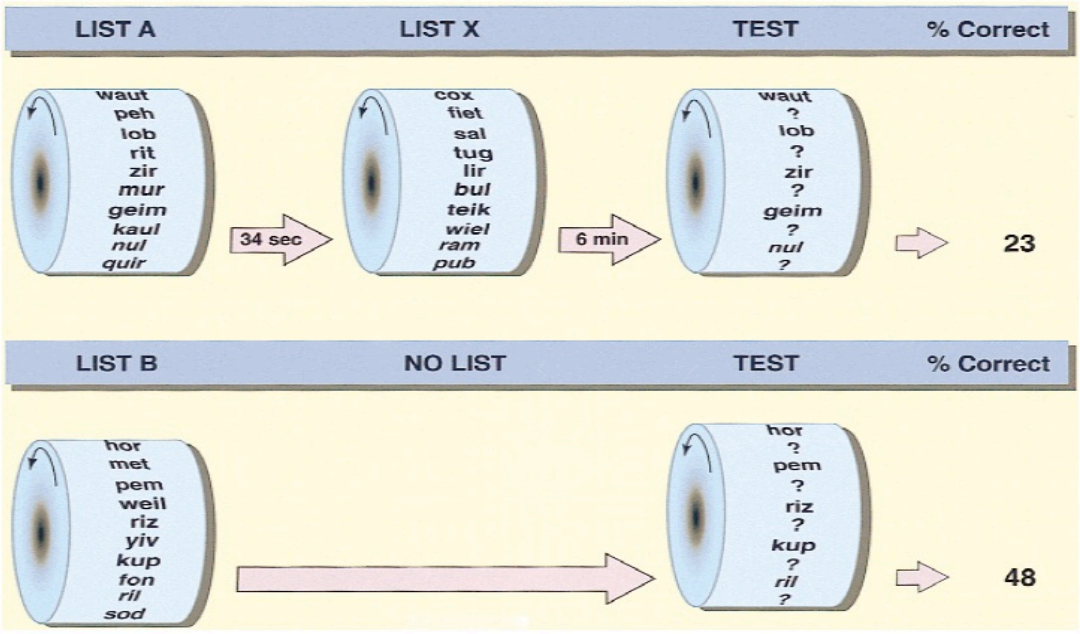

Figure 1: Schematic by Lechner et al. (1999) of tasks in the Müller and Pilzecker (1900) study. Each list (lists "A" and "X") contained a pair of nonsense words where one acted as a cue and the other acted as the item of retrieval. Participants were tested on list A at a later time where the cue was presented and the recollection paired nonsense word was tested. Participants who were exposed to the interpolated list X had a poorer recollection of list A than those were not.

Müller and Pilzecker (1900) also showed that the interfering material does not need to be similar to the original learning experience to have an effect on the recall of the initially learnt list. Instead of subjecting participants to a second list of paired nonsense words, they were asked to observe three paintings of landscapes and provide a verbal description of their observations to the experimenter for thirty seconds for each painting. As with the previously discussed experiment, there was a control group that were not subjected to an interference condition, see Figure 2 for a schematic representation of this experiment. Participants in the experimental group again performed worse than the control group. This finding suggests that the exertion of mental effort is sufficient to have a detrimental effort on the recollection of a previously learnt experience.

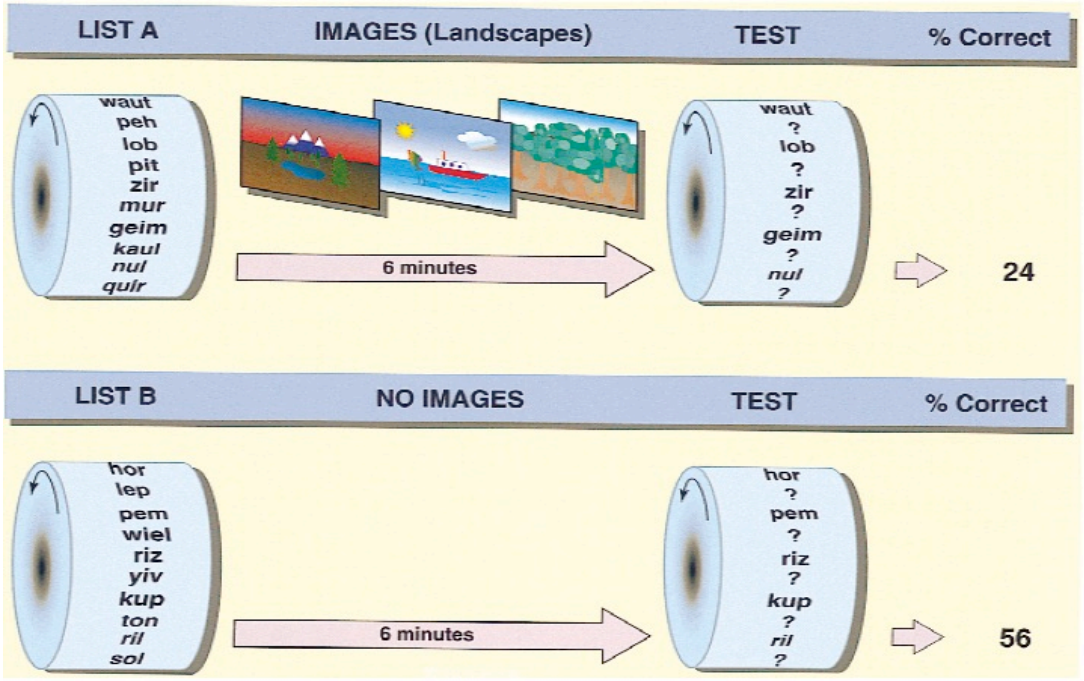

Figure 2: Another schematic by Lechner et al. (1999) of tasks in the Müller and Pilzecker (1900) study. All participants were subjected to learning "list A" that contained a pair of nonsense words where one aced as a cue and the other as the item of retention during test. All participants were subjected to the test session 6 minutes after learning "list A". The experimental group additionally underwent an interpolated session of observing and verbalizing the features of landscapes. This lead to poorer recall of the cue-associated word item.

They further investigated whether the time lag between the initial learning and the interfering material had differential effects of RI (Dewar et al., 2007; Müller and Pilzecker, 1900; Lechner et al., 1999; Wixted, 2004). During this experiment, participants learnt the nonsense word syllables, and were provided the interfering list either 17 seconds or six minutes after the initial learning experience. Those who received the interfering material after 17 seconds performed more poorly (28% recall) than those who received it after six minutes (49% recall). This suggests that a recently formed memory is more susceptible to RI than an older one. Müller and Pilzecker (1900) suggested that newly formed memories undergo a process they termed "consolidation" that makes the memory resistant to interference over time. They further suggested that non-task related mental exertions that take place during this time disrupts memory consolidation and in turn results in poorer performance during recall. The effect of RI discussed so far have been exacerbated in patients that suffered brain damage, particularly in the hippocampal area.

1.2 Memory Disruptions in Patients with Neurological Damage

Henry Gustav Molaison (HM), a patient who suffered from temporal lobe epilepsy, underwent a bilateral medial temporal lobe resection in order to remove the source of the seizures (Scoville & Milner, 1957). This lobotomy also included adjacent structures such as the hippocampus and the amygdala. Post-operative tests revealed that he suffered profound anterograde amnesia and some retrograde amnesia. The former meaning that he was unable to store newly learnt declarative material in long term memory (LTM), and the latter meaning that he lost some memories stored up to three years leading up to his operation. He was not different post-operatively in terms of his personality and intelligence with a slight improvement in the latter.

Scoville and Milner (1957) also reported similar traits in other patients who underwent similar lobotomies to patient HM. One most common trait was the complete forgetfulness of a recently learnt task after the mere introduction of a subsequent task and the severity of this condition was directly correlated with the amount of bilateral hippocampal sectioning. This suggests that the hippocampus is critically involved in the consolidation process of newly formed memories in humans. This was further supported by studies done in monkeys where bilateral hippocampal lesions led to memory retention deficits (Squire, 1992).

It therefore comes as no surprise that RI studies in rodents have typically focused on hippocampal-dependent learning experiences such as contextual memory. As mentioned earlier, this project aims to use specific findings from the animal literature that focuses on hippocampus-dependent learning experiences and to apply these findings to an appetitively-motivated learning experiences that involves brain areas like the nucleus accumbens (NAc) and do not primarily involve the hippocampus (Corbit & Balleine, 2000).

1.3 Retroactive Interference in Rodents

Following up on the evidence in human and monkey literature, studies aimed to produce RI in hippocampal-dependent tasks in rodents (Jarrard & Elmes, 1982). However, early attempts resulted in failure to induce RI in rats using spatial memory tasks (Beatty & Shavalia, 1980; Maki, Brokofsky, & Berg, 1979). Maki et al. (1979) subjected rats to an 8-arm radial arm maze. This maze consisted of an octagonal central platform where each side of this platform is an entryway to 8 identical arms radiating from the platform. At the end of each arm contained a food well that was baited with food pellets. Extramaze cues (such as furniture in the experimental room) were available for rats to orient themselves within the maze and hungry rats were allowed to explore all 8 radial arms and retrieve the food rewards during the session.

Rats were subjected to different interfering conditions for approximately 2 minutes after the first 4 radial arm entries (4 trials) were made. These interfering conditions included both distracting stimuli such as different lighting conditions (lights on/ lights off) or varying light intensities, or presenting white noise, providing food in the central platform, or removal from the experimental room during the 2-minute period. After being subjected to these interfering conditions, rats were replaced into the central platform of the radial arm maze and arm exploration was recorded to test for errors such as re-entries into the same arm (or already-visited arm). Results showed no effect of errors as a result of rats undergoing these distracting, interpolated conditions.

Maki et al. (1979) further studied whether the introduction of rats into a different, 4-arm radial arm maze was sufficient to produce retroactive interference. The number of errors made by rats undergoing this condition was not different from those made by the control group of rats that stayed in the central platform for the equivalent period of time.

Together, these results indicate that spatial memory in this paradigm is resistant to retroactive interference during short delay intervals.

Beatty and Shavalia (1980) subsequently attempted to increase the effectiveness of the interfering condition on spatial memory by building on the previously discussed study (Maki et al., 1979). The methods employed here were very similar to those in Maki et al. (1979) but the time between exploring the first four arms and the second four arms were extended to 4 hours instead of the two minutes used in the previous study (Maki et al., 1979). After the initial 4 radial arm entries rats were either returned to their home cages for 4 hours before being reintroduced to the radial arm maze (control group) or subjected to another 8 arm radial arm maze exploration session at varying times a (0, 5, 15, 30, 60, 120, 230 minutes) after the first four trials elapsed. However, the interfering conditions had no detectable effect on RI compared to the control condition. Given these findings, Beatty and Shavalia (1980) concluded that spatial memory in the radial arm maze is highly resistant to RI.

Although these early experiments showed no effect of RI, it must be noted that the method employed was conceptually different to those used to study RI in humans. In the human literature, participants were conventionally taught some material (pair of nonsense words) and asked to reproduce the syllable in response to a cue. The interfering material is often a novel learning experience unlike spatial tasks (four-arm radial maze) used in the formally discussed experiments. While the distraction tasks in Maki et al. (1979) were novel, they were still not new learning experiences.

With these caveats in mind, Jarrard and Elmes (1982) found a way to produce RI in a spatial learning task and investigated the effect of hippocampal lesions on their RI task. Jarrard and Elmes (1982) subjected rats to a learning experience in a 12-arm radial arm maze

where a unique set of four out of the 12 arms were baited with food reward. Rats were then either subjected to the interfering task where a different set of 4 arms were baited with the food reward or the non-interfering control task where rats were placed in a 4-arm maze that was baited with the food reward in all arms. Once the rats underwent both the interfering and control conditions they were further segregated in terms of the surgical protocol. Rats were divided into those that either received bilateral hippocampal lesions, cortical control lesions or un-operated controls. All rats were subjected to the retraining of the initially learnt task

Results showed that rats in the control conditions on average fared much better in relearning than those in the hippocampal lesion conditions. Rats that received the interference-control condition also fared better on average than those in the interference condition. Furthermore, task 2 related probes were significantly higher in the experimental condition than the control interference condition.

Rats that underwent hippocampectomy showed no difference in terms of the type of error they made but displayed significantly more errors in general. Hippocampectomy rats also committed more working memory errors where they revisited the same arm that was visited earlier in the trial suggesting problems with updating new spatial information. Rats were also not different in terms of other behaviours in the maze, for example, rats running speed was normal across surgical groups and they were not different in terms of food consumption behaviour. This suggests that it was only the contextual memory that was profoundly impaired due to hippocampectomy. These results are consistent with reports of human memory deficiencies in patients with hippocampal damage where there was a profound impairment in the updating of new memories as well as impaired memories

recently formed before hippocampal damage while other cognitive processes were spared (Scoville & Milner, 1957; Weiskrantz, 1987).

Findings above suggest that a subsequent spatial learning experience in the same context produced RI in these rats which might indicate that the hippocampus was overloaded by being subjected to the interpolated learning experience. Furthermore, permanent lesions produce profound impairments in contextual memory, thus making it difficult to interpret whether new learning or expression of a previously learnt experience is impaired by hippocampal lesion. Temporary/reversible inactivation of the hippocampus therefore helps tease these functions apart.

1.4 Temporary Neural Disruption in the Hippocampus

Butterley, Petroccione, and Smith (2012) investigated the effect of contextual cues on an odour related retroactive interference task. Briefly, rats were trained in two odour discrimination problems (named “list 1” and “list 2”) in either the same or different contexts as shown in Figure 3. In this procedure, hungry rats were first presented with two food wells in a room while both wells were covered with bedding (list 1). Only one of the two wells contained a food reward and both wells were covered in distinct odours. Rats were subjected to 8 different pairs of 16 distinct odours where one was reinforced and the other was not. Rats underwent this discrimination condition until reaching a criterion of 90% correct odour related choices across two consecutive sessions.

Rats were then subjected to the interference condition (list 2) where they experienced it in either the same context as they learnt list 1 or in a different context to list 1. Half of the odours experienced in list 2 were used in list 1 and were paired with an odour that was not experienced during list 1 (see Figure 3 for details). Furthermore, if the common odour was reinforced in list 1 it was unreinforced in list 2 and vice versa.

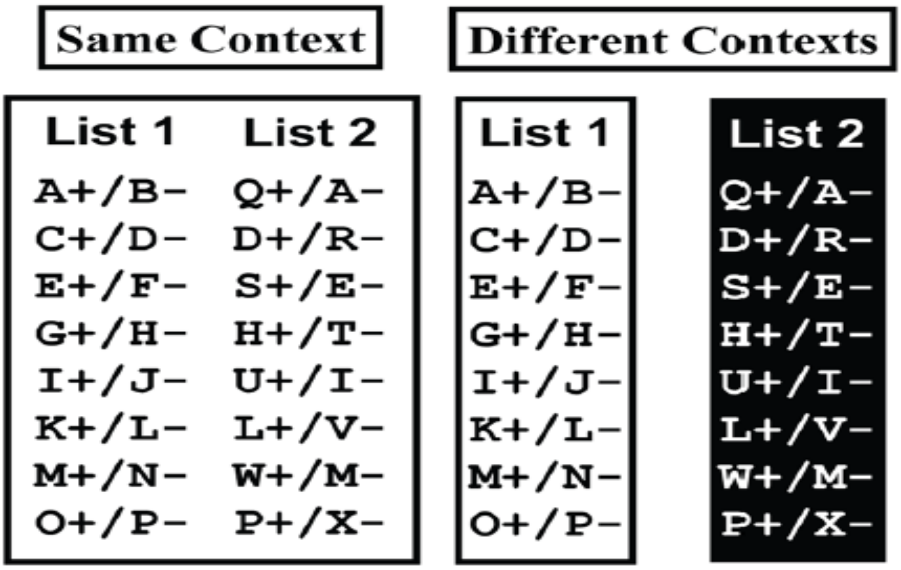

Figure 3: Schematic by Smith and Bulkin (2014) representing the learning undergone by rats in both list 1 and 2 in either the same (left) or different contexts (right) in the Butterley et al. (2012) study.

Half the rats undergoing list 2 training in either context received muscimol injections into the hippocampus while the other half received physiological saline into the hippocampus. Therefore, rats were divided into four groups; the group that received saline injections that either underwent list 2 training in the same or different contexts and the group that received muscimol injection while training in list 2 in either the same or different contexts.

Rats in the saline group showed a greater amount of RI when undergoing list 2 learning in the same context than those in a different context as shown in Figure 4. This effect of context on alleviating RI was not apparent in the muscimol group. Furthermore, when rats were subjected to an easier list 2 in a different context where there was no overlap in odours from list 1, there was no difference in performance between rats that that were subjected to muscimol and saline. This suggests that the hippocampus is recruited to differentiate more difficult tasks by associating a context to each task, a concept that is extended to object recognition memories (Martinez, Villar, Ballarini, & Viola, 2014).

While Butterly et al. (2012) accentuated interference that is prevented by the normal functioning hippocampus, Martinez et al. (2014) first designed a behavioural paradigm that produced RI by overloading the hippocampus and further found that silencing the hippocampus during the second learning experience diminished this effect of RI.

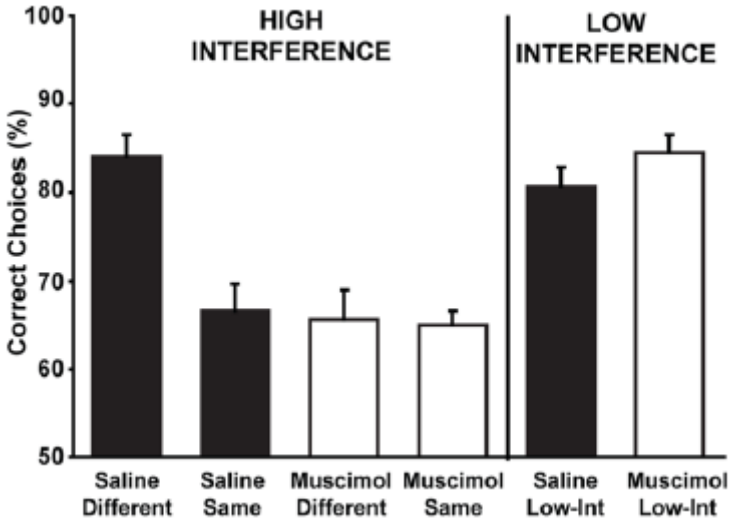

Figure 4: Behavioural results of rats in Butterley et al. (2012) (from Smith and Bulkin, 2014) Performance of rats in both the high (overlapping smells between Lists 1 and 2) and low (no overlap of smells) interference groups and the differential effect of hippocampal silencing (using muscimol) when administered as rats learn list 2 in a different context when compared to those in the control condition.

In this paradigm, rats were first introduced to two identical objects (objects 1) in a particular context (room 1) and were reintroduced into room 1 for a test session 24 hours later. When revisiting room 1, rats were exposed again to two objects where one remained the same as the previously introduced object and the other was a new object. During the test session, rats were monitored for signs of exploration towards either objects and found that rats explored the novel object more than the previously encountered object. In the interfering condition, rats were introduced to a second context (room 2) one hour after the introduction of objects 1 in room 1. This time the rats were introduced to two different identical objects (objects 2). 24 hours later, rats were placed in room one with one object

from context one and one from context 2. As shown in Figure 5, the exploration analysis showed no difference in exploration between the two objects. Since rats have an innate tendency to explore new objects within a particular context compared to objects that have already been explored in the context, it was expected that rats would explore the object 2 more than the object 1 in context 1 during the test session. When rats were placed in room 2 instead of room 1 during the test session, exploration was greater for object one in room 2. This is consistent with earlier finding that rats tend to explore novel objects in a familiar context.

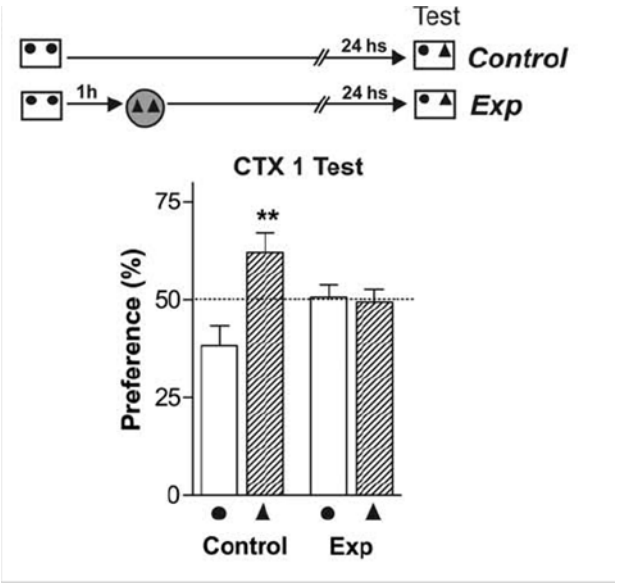

Figure 5: Effect of undergoing an interpolated learning experience on the exploration of the previously explored object in the context and the one explored in the different context (from Martinez et al., 2014).

The authors suggested that exploring object 2 in context 2 retroactively interfered with the memory of experiencing object 1 in context 1 and so the rats explore the two objects for equal amounts of time.

In order to further test whether the interference occurred as a result of disruption in memory consolidation, rats were introduced to the test session in context 1, 30 minutes after experiencing objects 2 in room 2. The amount of exploration of object 2 was

significantly higher during the test session. The same effect was seen with rats in the non-RI control condition, thus suggesting that the interference is on consolidation of memory rather than interference of the original, short-term memory trace as seen in Figure 6A.

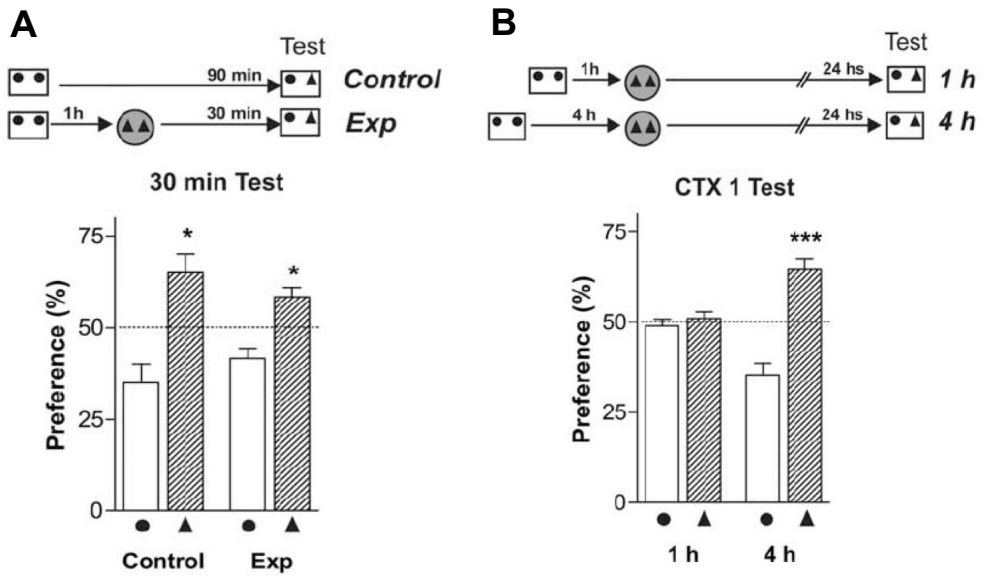

Figure 6: Differential effect of the interpolated learning experiences at different time gaps from test and from the initial experience (from Martinez et al., 2014). (A) Rats showed a diminished effect of RI when decreasing temporal gaps between the interpolated experience and the test session. (B) The effect of RI was also diminished when increasing the time between the two initial learning experiences to 4 hours.

In another condition, the time between experiencing the interfering condition (objects 2 in room 2) and the original experience (objects 1 and room 1) was extended from one hour to four hours while the time between the interpolated learning condition and test condition remained (objects 1 and 2 in room 1) 24 hours. Rats explored object 2 more than object 1 during the test condition, thus suggesting that the effect of interference is not seen when the interpolated learning condition falls outside the window of consolidation of the initial memory trace. This is also shown in Figure 6B.

Using the temporal parameters of RI of one hour and 24 hours, Martinez et al. (2014) infused the dorsal hippocampus with muscimol bilaterally 15 minutes before experiencing the second task. This disrupted the normal functioning of the hippocampus while

experiencing the interfering task. When exploration percentages were tested with objects one and 2 in room 1, rats explored object 2 more than object 1. In a control condition where rats received bilateral saline infusion instead of muscimol, exploration was not different across the two objects as seen in Figure 7. Therefore, the effect of RI was alleviated by virtue of temporary hippocampal activity disruption during the interpolated learning condition.

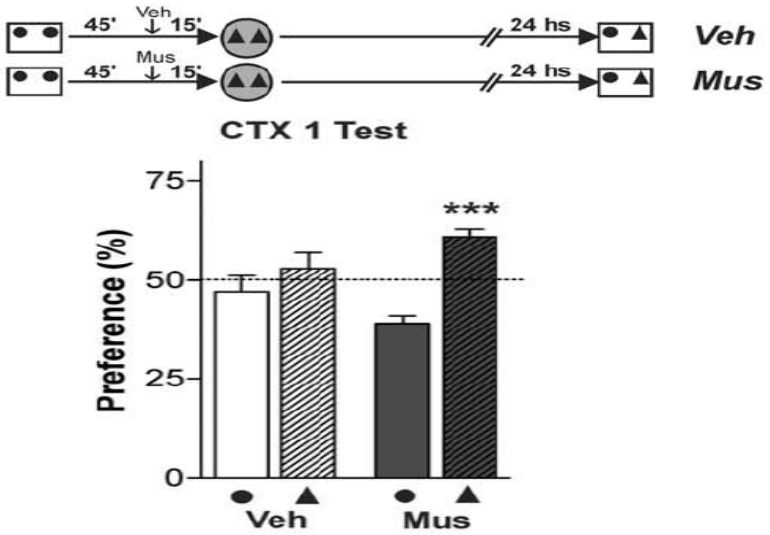

Figure 7: Effect of neuronal silencing of the hippocampus during the interpolated condition (from Martinez et al., 2014). Silencing the hippocampal activity using Muscimol (Mus) during the interpolated learning experience alleviated the effect of RI. This was not seen in the group that received saline (Veh).

In summary, the studies discussed above suggest that tasks involving the hippocampus such as the radial arm maze and object to context recognition memories are resistant to RI during short term retention periods but are susceptible to its effects after longer intervals. Furthermore, irreversible hippocampal lesions led to more profound deficiencies in relearning an already learnt task compared to un-lesioned rats that underwent a retroactive interference condition. Temporary lesions of the hippocampus during the interpolated learning condition alleviated the effect of RI.

Although studies above used a multitude of different paradigms such as object recognition, odour discrimination and spatial navigation to investigate RI, the primary manipulation has been on the contextual memory of these tasks instead of manipulating

tasks themselves. Furthermore, manipulation of the hippocampus spared the learning of the odour discrimination task while affecting the contextual memory. These tasks have also relied on overlapping task requirements between the initial task and the interpolated learning experience in order to produce RI. Therefore, it is not clear whether the effect of RI is due to some form of confusion during retrieval or whether the second (interpolated) learning experience is indeed preventing the consolidation of the memory during the initial learning experience.

The present study therefore aimed to test whether RI can be induced in tasks without intersecting task requirements and to probe whether manipulating brain regions responsible for the task themselves can have an effect on the RI. The temporal parameters assigned to the hippocampal studies by Martinez et al. (2014) was used as a guide to test for RI in an appetitive operant discrimination task by an interfering, classical conditioning task. As mentioned earlier, these learning experiences have different task demands but use the same underlying brain circuits such as the NAc. Furthermore, lesions and reversible inactivation of the NAc core disrupts the learning, consolidation and expression of both operant and classical conditioning in a similar manner to how these manipulations affect contextual memories when applied to the hippocampus.

1.5 A-O and S-O Associations and the Nucleus Accumbens (Nac)

Balleine and Killcross (1994) examined the effect of irreversible bilateral NAc lesions on instrumental (A-O) behaviour. Rats with cannula implantation aimed at the nucleus accumbens underwent multiple training sessions where they learnt to press a lever for food pellets. After training, rats were either subjected to accumbens lesion (using Ibotenic acid) or the sham (control).

Following recovery from lesion, rats were subjected to retraining of the aforementioned lever press task. Relative to a pre-lesion baseline, rats in the sham (control) lesion group were performing at a comparable level by the end of the post-lesion retraining session. However, as seen in Figure 8 rats in the lesion group did not recover their former level of performance. The rate of head entries was also recovered by the last session of retraining in the control group but was unrecovered in the experimental group. Although, the same trend was seen in an independent group that were trained to press a lever for sucrose solution, the effect was diminished in comparison to the pellet group. These results suggest that the nucleus accumbens is important in the performance of an A-O task.

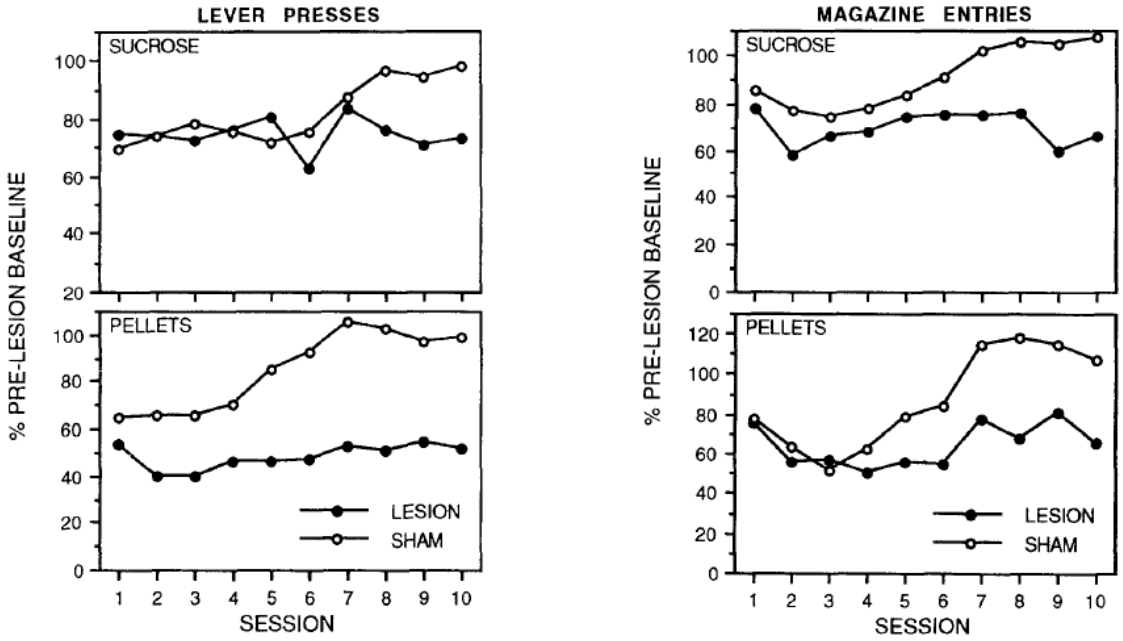

Figure 8: Head entry and lever presses performances for differential rewards (pellets and sucrose) relative to a pre-lesion baseline (from Balleine and Killcross, 1994). Rats showed a diminished level of lever press and head entry performance relative to a pre lesion baseline for both food pellets and sucrose rewards compared to a sham control.

Using a similar protocol, Parkinson et al. (1999) found a deficit of accumbens lesion in an S-O Pavlovian conditioning task. They examined whether bilateral lesions of both the NAc core and the shell had differential effects on Pavlovian (S-O) conditioning. Rats underwent

multiple Pavlovian training sessions leading up to the lesion procedure. In each trial, rats were provided access to sucrose solution after the presentation of a CS stimulus.

After the initial behavioural training, rats were subjected to either NAc core or shell lesions using ibotenic acid or sham (control) lesions. Once recovered form from surgery, rats were reintroduced to 4 classical conditioning sessions to test for any deficiencies in performances. Results showed a significant increase in food magazine head entries during CS presentation relative to the VI interval for both sham and NAc shell lesion groups over successive post-operative sessions. However, this was impaired in the core lesion group of rats. Figure 9 shows the ratio of mean head entries during the CS presentation interval relative to CS+VI interval for four sessions before and after surgery.

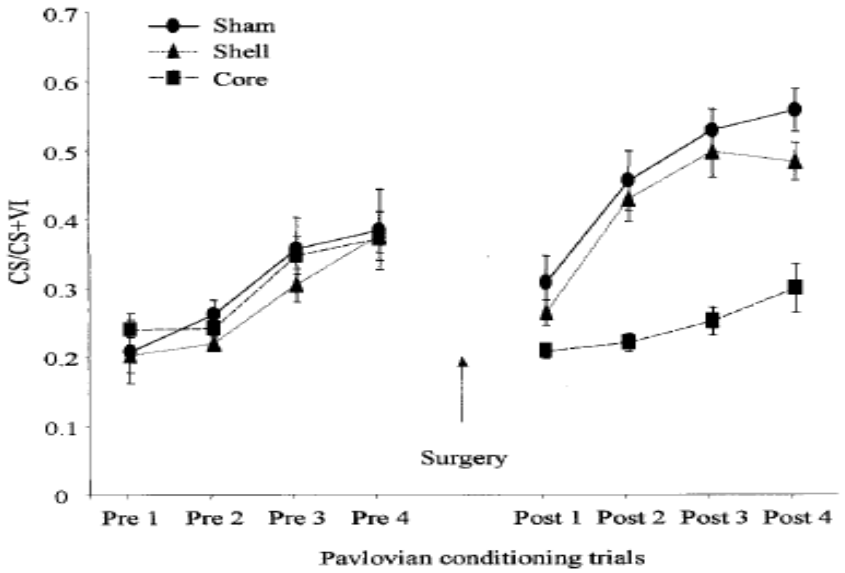

Figure 9: Differential effect of NAc core and shell lesion on the performance of a classical conditioning task (from Parkinson et al., 1999). Diminished performance in a classical conditioning procedure when the NAc core is ablated but not in the sham control or NAc shell ablation.

Taken together, following lesions in the accumbens, rats were unable to recover their pre-lesion level of performance in both A-O and S-O tasks. This phenomenon is paralleled by the findings of Jarrard and Elmes (1982) who showed profound deficiencies in relearning a spatial discrimination task that involves the hippocampus. While permanent lesions also show profound learning impairments on A-O and S-O tasks it is difficult to decipher whether

this deficit is due to learning, storage, retrieval or performance of A-O and S-O associations. Therefore, timely reversible disruptions of these brain areas help in teasing these functions apart. For example, disrupting the neural activity while rats undergo a learning experience might reveal whether it affects the initial learning of the task and reversible disruptions immediately after a learning experience might reveal whether the brain region is important for the consolidation of a recently learnt task.

Kelley et al. (1997) tested the effects of AP-5 application in either the nucleus accumbens core or shell during both appetitive instrumental and Pavlovian learning tasks. AP-5, a competitive N-Methyl-D-Aspartate (NMDA) receptor antagonist has produced marked deficits in spatial learning when applied to the hippocampus (Kawabe, Ichitani, & Iwasaki, 1998). Since the medium spiny neurons of the NAc were also found to express these NMDA receptors, Kelley et al. (1997) tested whether similar deficits occur in instrumental and pavlovian learning under the influence of AP-5.

Rats were first bilaterally implanted with cannulae aimed at either the core or the shell sub region of the NAc. After recovery from surgery, rats underwent ten, 15-minute sessions of operant discrimination procedure where one of the two levers were reinforced with a pellet reward while pressing the other produced no consequence. For the first 4 sessions, rats in both core and shell implant group received either AP-5 infusions or control infusions immediately before the start of the session. Pre-session infusions were ceased for the next 5 sessions (sessions 5-9) and were reinstated on the last (10th) session and the number of correct and incorrect lever presses and head entries were recorded.

As shown in Figure 10, rats in the AP-5 infusion group were unable to discriminate between the correct and incorrect lever for the first four sessions. Their control counterparts started discriminating between the two levers by the third session. Once pre-session

infusions of AP-5 were ceased, rats gradually learnt to discriminate between the reinforced and unreinforced levers and reintroduction of AP-5 into the core on the 10th session did not affect the learnt discrimination. This trend was also reflected in the amount of head entries made by the rats in each session. These results suggest that AP-5 prevented the initial learning of an operant discrimination task but spared performance of a learnt operant discrimination task.

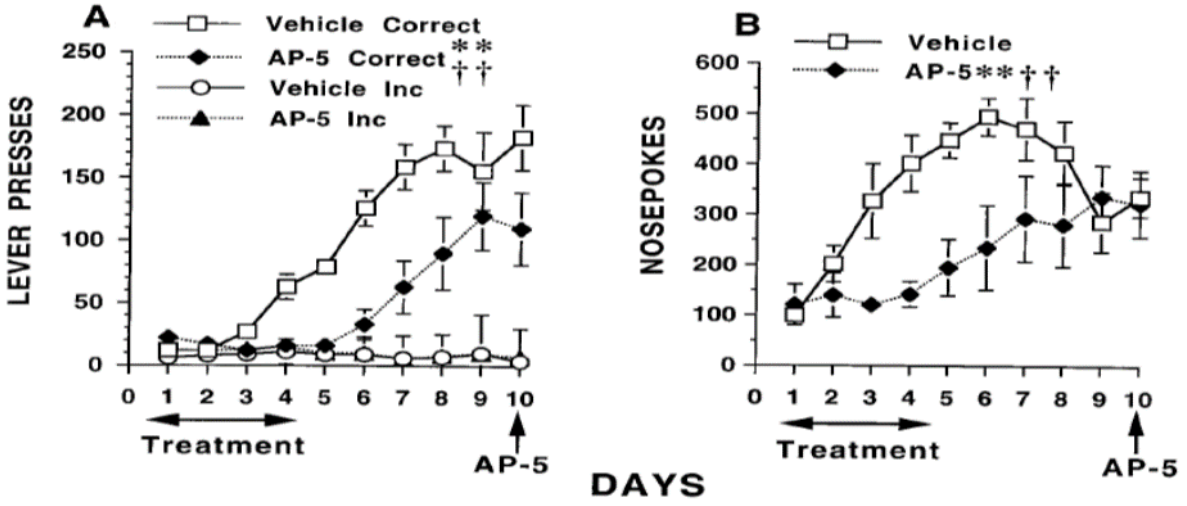

Figure 10: Learning and post-learning effect of pre-session, intra accumbens infusion of AP-5 in operant discrimination (lever presses) and head entries (nose pokes) (from Kelley et al., 1997). Pre-session accumbens disruption (using AP-5) impaired the initial acquisition of an operant discrimination but did not impair the performance of an already learnt operant discrimination behaviour (left). The same trend was seen in head entry behaviour (right).

Importantly, these results were shown to not be due to any effects of AP 5 on any other aspects of task performance. This included behaviour such as feeding, locomotor performance, or general motivation.

Kelley et al. (1997) further tested whether pre-session intra accumbens core infusions of AP-5 affected the learning of a Pavlovian conditioning paradigm. Rats underwent 45 trails in each of the 5 sessions. In each trial, rats were presented with a compound stimulus of a visual and auditory stimulus that predicted pellet delivery. The visual stimulus

was a 3 second illumination of a red light with the houselight turned off and the auditory stimulus was the sound of the pellet dispenser that lasted a second. The number of head entries were recorded during each session where a greater number of head entries during the CS presentation suggests pairing of the stimulus with the pellet reward.

Results showed that the number of pre-CS entries were not different in all 5 sessions across the experimental and the control group. However, the number of CS related head entries were lower in the experimental group than the control group between sessions 2 to 5. This suggests that pre-session infusion of AP-5 impaired the learning of an S-O association. Kelley et al. (1997) therefore provided evidence that reversible disruption of accumbens activity (the core sub region in particular) during the learning phase impairs the development of both A-O and S-O associations.

As previously defined by Müller and Pilzecker (1900), recently formed memories undergo a process called consolidation in order to render these newly formed memories resistant to interference. Neurobiologists have found that the disruption of hippocampal brain activity using anisomycin after learning of a new task negatively affects the retention at a later time (Naghdi, Majlessi, & Bozorgmehr, 2003; Remaud et al., 2014; Rossato et al., 2007). Building on these findings, Hernandez et al. (2002) tested whether anisomycin administered in the NAc had a similar effect on an operant discrimination task. Rats were subjected to the same operant discrimination protocol as the previously described study by Kelley et al. (1997). Instead of pre session application of AP-5 rats received a post session application of anisomycin into the accumbens core (Hernandez et al., 2002).

When administered a low concentration of anisomycin into the accumbens core, rats showed some evidence of lever discrimination by the final session of anisomycin treatment. However, rats receiving a high concentration of anisomycin showed no signs of learning in

the days of post session infusion. Rats subsequently showed evidence of lever discrimination in sessions following the cessation of anisomycin infusion as seen in Figure 11A. This deficit was not seen in an independent group where rats received infusions of anisomycin into the medial shell at a high concentration (Figure 11B), thus suggesting that the accumbens core but not the shell is critical for A-O memory consolidation.

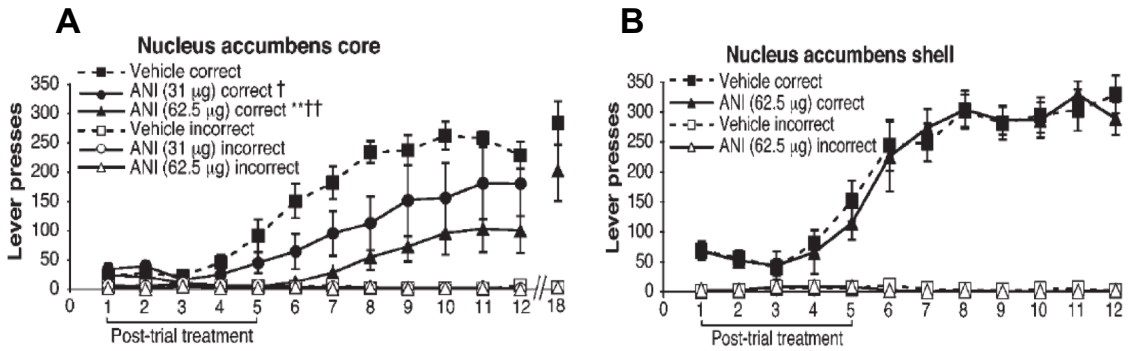

Figure 11: Differential effect of post session NAc disruption using Anisomycin in the NAc core and shell sub-regions (from Hernandez et al., 2002).(A) Rats showed no learning and diminished level of learning when high and low concentration of anisomycin was administered into the NAc core. (B) There was no effect of post-session infusion of anisomycin in high concentration when infused into the accumbens shell.

Hernandez et al. (2002) further investigated whether NAc core disruption using anisomycin had an effect on learning at extended post-session infusion times. Rats were infused with the high concentration of anisomycin either 2 or four hours after each session and the results showed no effect on learning compared with their control counterparts as shown in Figure 12 Furthermore, performance was unaffected when rats were given anisomycin after rats had reached an asymptotic level of discrimination between the two levers.

This temporal effect of anisomycin was consistent with the aforementioned RI study (Martinez et al., 2014). Rats that underwent the interpolated learning condition one hour but not four hours after the initial learning experience showed a behavioural effect of RI. This raises the question as to whether a behaviourally elicited activity of the NAc core that is

non-task related to an A-O learning experience can also have a disruptive effect on the consolidation of a learnt A-O task. We therefore tested whether subjecting rats to an accumbens dependent task (such as an S-O task) that is behaviourally non-conflicting with an A-O task interfered with the expression of the learnt A-O association at a later time.

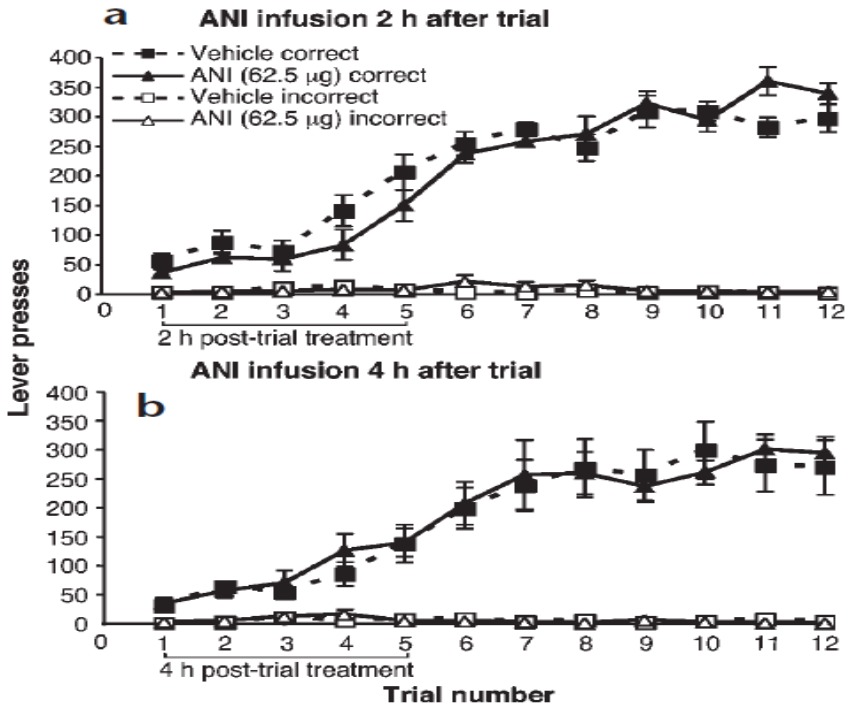

Figure 12: Effect of delayed, post-session disruption of NAc core showed no effect of disruption in learning compared to control (from Hernandez et al., 2002). Delaying the post-session infusion of anisomycin into the NAc core had no effect on learning the operant discrimination task when administered either 2 hours (top graph) or four hours (bottom graph).

1.6 Current study

The current study used the temporal parameters established for RI by Martinez et al. (2014) and applied it to an A-O and S-O related RI task. We first developed an RI paradigm using an operant task as the initial learning experience and a classical conditioning task as the putatively interfering interpolated learning experience. One group of rats (group 1) were first subjected to a temporal gap of 1 hour between the initial (A-O) and interpolated (S-O) learning experiences. After a 24 hour period of hiatus, rats were subjected to a probe version of the initial, A-O learning experience. The performance during the probe condition was compared to a group (group 2) that did not undergo the interpolated learning

experience. We predicted that, similar to the results found by Martinez et al. (2014), Group one would show RI, while Group 2 would not.

A further test was conducted to investigate whether the RI produced by the S-O learning experience acted on the short term memory (STM) of the initially learnt A-O task or the long term memory (LTM) of the initially learnt experience. This was assessed by testing another group of rats (Group 3) in which the hour interval between the initial and the interpolated learning experience was maintained while the time between the interpolated learning experience and the probe session was decreased. The performance of rats in this group during T1-P was hypothesized to be better than the rats in group 1 as a similar result was found by Martinez et al. (2014).

A final group of rats were subjected to an extended temporal gap between the initial and the interpolated learning experience while maintaining the time between the S-O learning experience and the probe session at 24 hours. This was done to test whether the S-O learning experience had an effect on the initially learnt A-O learning experience outside the consolidation time window of 2 hours found earlier (Hernandez et al., 2002).

To test whether the RI found in the first experiment was dependent on activation of the nucleus accumbens during the interfering interpolated learning experience, we conducted a second experiment in which rats were subjected to neural silencing in the nucleus accumbens core region using the designer receptors exclusively activated by designer drug (DREADD) technology during the interpolated, S-O learning experience (Armbruster, Li, Pausch, Herlitze, & Roth, 2007; Whissell, Tohyama, & Martin, 2016; Zhu & Roth, 2014). Since Martinez et al. (2014) found that silencing the neural activity of the hippocampus reduced the effect of RI in their paradigm, the current study was also

hypothesized to produce a similar effect by silencing the activity of the NAc core during the interpolated learning experience.

2 Methods

2.1 Equipment

Ten operant chambers (Med associates, St. Albans, VT: Model ENV- 008w) with the internal dimensions of 30.5cm (length) x 24.1cm (width) x 21.0cm (height) were used to conduct the following behavioural procedures. In one of four walls, the operant chambers consisted of a food hopper with identical retractable levers on either side. Head entries into the food hopper were recorded by an infrared photocell detector and a house light and an audio speaker were fixed on the opposite wall. The houselight provided illumination and the speakers produced a 90-dB noise at a pitch of 2500Hz. The floor of the operant chamber was made up of cylinders that are spaced 0.87 cm apart.

Each operant chamber was housed within cupboards that blocked ambient light and attenuated ambient noise. 72 dB of white noise was generated by a fan in each operant chamber to further attenuate white noise. Operant chamber programming and data recording was performed using the MED-PC IV software.

2.2 Subjects

52 long Evans rats were grouped two or three to a cage. In order to motivate the rats during experimental sessions, they were food restricted and maintained at 85% of their free feeding (base) weight. Water was provided ad-libidum throughout the experimental procedure. All animal handling and use complied with the Animal Welfare Act 1991, and experiments were conducted under the approval of the University of Otago Animal Ethics Committee (Protocol number: 17/16)

2.3 Behavioural Protocol

2.3.1 Pellet Exposure

24 hours before behavioural training began, rats were introduced to the food pellets that were later used as rewards during operant chamber procedures. Food pellets in small trays were placed in the rats' home cages during their normal feeding time. Rats were monitored until each rat within the home cage consumed at least one of these pellets before being provided their usual rat chow 15 minutes later. These trays were removed before the preliminary training began the following day.

2.4 Preliminary Behavioural Training

2.4.1 Pellet training

This procedure was designed for the rats to learn to retrieve food rewards from the food hopper. In a single session, 60 pellets were dispensed into the food hopper with an average inter-trial interval of 30 seconds between them. The number of head entries into the food hopper was recorded during the session. Once rats reliably retrieved and consumed at least 50 out of the sixty dispensed food pellets, they moved on to the lever press training procedure. All rats achieved this criterion within 5 pellet training sessions.

2.4.2 Lever press training

In this procedure, rats were taught to press levers in order to receive a food reward. Only one of the two levers was extended for the rats to press at a given time. Each lever was extended for 10 seconds in a pseudorandom order. If the extended lever was pressed within 10 seconds, the levers retracted, and a single food pellet was dispensed into the food hopper. If the lever was not pressed, the lever retracted after 10 seconds of extension and a food pellet was dispensed into the hopper. Rats were provided 60 trials in total, half the trials were extension of the right lever and the other half was the extension of the left lever.

There was an average inter trial interval of 30 seconds. Rats took between 4 to 5 sessions before pressing levers on at least 50 out of 60 trials.

2.5 Experiment 1

As mentioned earlier, a group of rats were subjected to the initial A-O learning experience and the interpolated S-O task before being subjected to the probe session of the initially learnt task. The performance of these rats were compared to those of another group that did not undergo the interpolated (S-O) learning experience.

Furthermore, two additional groups were tested with varying delays between the A-O, S-O and the probe conditions to characterize the nature of the obtained RI. The following section provides specific details on these behavioural conditions and the temporal parameters of these conditions.

2.5.1 Behavioural conditions.

Task 1 Operant, A-O discrimination protocol (T1). This was a single 50-minute session that all rats in experiments 1 and 2 underwent 24 hours after successful lever press training.

Both levers were extended throughout the session where pressing the active lever resulted in the dispensing of a single food pellet on a continuous reinforcement schedule. Pressing the inactive lever yielded no consequence. Active lever assignment was counterbalanced across all rats in each group and the sequence of active and inactive lever presses were recorded for the entire session.

Task 2 Pavlovian, S-O conditioning protocol (T2). For the groups of rats that underwent task 2 the protocol was as follows. Rats were subjected to 12 trials of the classical conditioning paradigm over 50 minutes. In each trial a tone was played for 10 seconds before food is delivered in the food hopper and each trial had an average inter trial interval of 240 seconds

with a range of 10.29 and 836.38 seconds. The number of head entries into the food hopper for the duration of the CS (10 seconds) and the equivalent time leading up to CS presentation was recorded for every trial.

Off-task condition (OT). Rats were placed in the operant chambers with the lights on for 50 minutes one hour after undergoing T1 but did not undergo any behavioural tasks during this time.

Task 1 Probe (T1-P). This was a 15-minute probe condition of T1 where both levers are extended simultaneously throughout the session. Pressing either lever yielded no food reward and the lever presses were recorded throughout the session. The temporal gap between T1-P exposure and T2 completion depended on group allocation and detailed descriptions and a schematic diagram (Figure 13) of rats' condition exposure and temporal gaps between conditions follow.

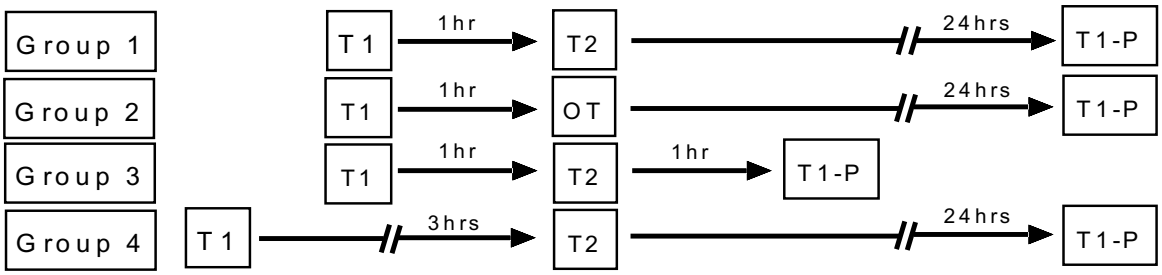

Figure 13: Schematic representation of conditions (T1, T2, OT and T1-P) that rats in each group underwent and the time gap between each condition during experiment 1.

2.5.1.1 Group 1

All experimental procedures took place in the same operant chambers for the same rats. As shown in Figure 13, rats in this group were first subjected to the T1 session, returned to the home cages for an hour of hiatus before being subjected to T2. Rats were left in the home cages for 24 hours before being subjected to the T1-P session.

2.5.1.2 Group 2

Rats in this group underwent T1 and T1-P with the same temporal distances between one another as group 1. Instead of being subjected to T2, rats were subjected to the OT condition.

2.5.1.3 Group 3

Rats in this group underwent the T1, T2 and T1-P conditions. The time gap between T2 and T1-P was reduced to one hour while the time gap between T1 and T2 remained one hour.

2.5.1.4 Group 4

Rats underwent the T1, T2 and T1-P conditions where the temporal gap between T1 and T2 were extended to three hours and the time gap between T2 and T1-P remained 24 hours.

2.6 Experiment 2

Neural silencing was achieved by introducing a mutated human muscarinic G-protein coupled receptors (GPCR) named hM4Di on the surface of the cells of the NAc core. These mutated receptors are irresponsive to the endogenous ligand acetylcholine and are instead activated by a small molecule called clozapine - N - oxide (CNO). CNO is a metabolite of clozapine that is otherwise biologically inert. CNO can also cross the blood brain barrier making it possible to activate these GPCRs using remote systemic injections such as intraperitoneal (IP) and subcutaneous (SC) injections.

Therefore, rats in this study underwent an initial neurosurgical procedure to insert the CPCR gene for hM4Di into the neuronal cells of the NAc core region. After the expression of these receptors on the cell surface, rats were subjected to the behavioural procedure that produced the most robust effect of RI. During this time, rats were administered with CNO to

activate the hM4Di receptors when rats experience the interpolated S-O learning experience. Upon activation, hM4Di causes the opening of inward rectifying potassium (K+) channels, leading to the hyperpolarizing of the host cells. This in turn decrease the likelihood of cell firing in the NAc core region during the interpolated S-O learning experience.

Due to previously reported effects of hM4Di and CNO independently (Manvich et al., 2018; MacLaren et al., 2016), there were three other control groups that have different combinations of control surgery and control IP injection. In order to test whether the expression of hM4Di itself has an effect on behaviour, a group of rats with hM4Di were subjected to a control IP injection of biological saline instead of CNO when undergoing the interpolated learning experience. Conversely, to test whether CNO had an effect on behaviour by itself, another group of rats received a control surgical procedure where GFP is expressed in the cells of NAc core without hM4Di. Finally, a separate group of rats received both control surgery (GFP) and IP injection (saline) to serve as a baseline for the other groups (Smith et al., 2016).

2.7 Procedure

2.7.1 Surgery

Thirty rats were anesthetised using isoflurane and were mounted onto the stereotaxic frame. 18 rats received 1 µl viral injection of AAV2-hSyn-HA-hM4D (Gi)-IRES-mCitrine (hM4Di) with the titre of 5.6×10^{12} particles/ml and the rest (n=12) received 1 µl viral injection of AAV2-hSyn-HA-EGFP (GFP) with the titre of 33×10^{12} particles/ml. Both viruses were purchased from the Gene therapy Center, Vector Core of the University of North Carolina. These injections were administered bilaterally to the NAc core region.

Flat-skull stereotaxic coordinates of A/P + 1.68, M/L +/- 2.0 and D/V -6.5 relative to bregma were used to aim at the NAc core and these coordinates were determined by referring to the "Rat Brain Atlas" (Paxinos, Koutcherov, Halliday, Watson, & Wang, 2007).

After surgery, Rats were provided with analgesics for two days, free fed and monitored for 7 days before being food restricted. These rats were then maintained at their 85% base weight and were exposed to the behavioural protocol after three post-operative weeks to allow viral expression to take place.

2.7.2 Behavioural protocol

Rats were subjected to the aforementioned, preliminary behavioural training procedure after the three-week period of postoperative rest. Rats were then allocated to groups and followed their behavioural procedures 24 hours after the successful completion of the lever press training task.

In Experiment 2, all rats experienced the same behavioural conditions and temporal parameters as group 1 in experiment 1. This group was chosen as it produced the clearest effect of RI (see results section for more detail). Group allocation for experiment 2 was based on the combination of their cerebral (hM4D1/GFP) and intraperitoneal injection (CNO/SAL). There was one experimental group (group 1) and three control groups (Groups 2, 3 and 4). Details of each group along with a schematic diagram (Figure 14) of the procedure is provided below.

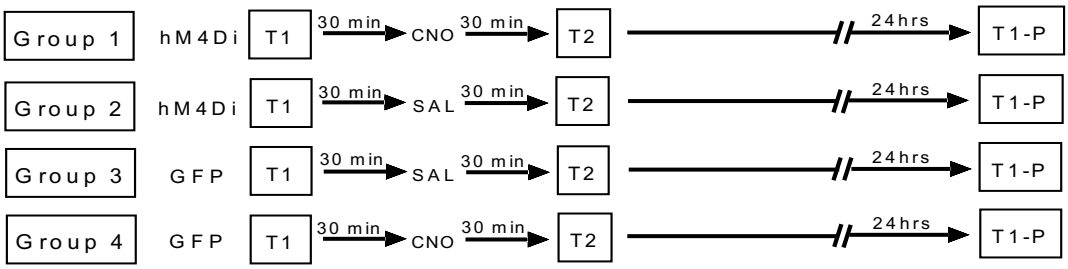

Figure 14: A schematic representation of experiment 2 showing the type of surgical injection in the NAcc (hM4Di/GFP) and the IP injection (CNO/SAL) between T1 and T2 in each group. It further depicts the operant chamber tasks (T1, T2 and T1-P) and the temporal gaps between the rats' exposure to them.

2.7.2.1 Group 1 (hM4Di + CNO).

Half of the rats that were injected hM4Di were allocated to this group. Rats underwent the same behavioural conditions as group 1 from experiment 1. In order to activate the hM4Di, they were administered an intraperitoneal injection of clozapine-N-Oxide (CNO) (received as a gift from the NIMH) with the concentration of 2 mg/Kg, 30 minutes before undertaking T2.

2.7.2.2 Group 2 (hM4Di + saline).

The remaining rats injected with hM4Di were allocated to this group. The behavioural procedures are the same as group except for the IP injection. These rats received an injection of saline (SAL) instead of receiving an injection of CNO.

2.7.2.3 Group 3 (GFP + CNO).

Half of the rats with bilateral GFP virus injection were allocated to this group. All rats in this group underwent the same behavioural procedure as group 1 where the IP injection of CNO (2 mg/Kg) was administered 30 minutes before being subjected to T2.

2.7.2.4 Group 4 (GFP + Saline).

Only 4 out of the remaining rats with bilateral GFP in the NAcc were allocated to this group as two rats did not survive the surgery. All rats underwent the same behavioural

procedure as group 1 where an IP injection of saline was administered before commencing T2.

2.7.3 Histology

Once behavioural procedures were complete, all rats from experiment 2 were deeply anesthetised with isoflurane and were transcardially perfused with 100 ml of saline and 100 ml of 10% formalin solution. Rat brains were removed and placed in a 10% formalin solution for 24 hours before being transferred to a solution of phosphate buffer (PB) with 30% sucrose. The phosphate buffer solution was custom made using sodium phosphate monobasic (i.e. NaH_2PO_4*H2O) and sodium phosphate dibasic (i.e. Na_2HPO_4*H2O) and was provided by Professor Cliff Abraham's laboratory at the University of Otago. Over the course of 3 to four days, the brain samples sunk to the bottom of the container, making it ready for sectioning. Sections at 40 µm thickness were made and stored in phosphate buffer solution before immunohistochemistry for slice visualisation.

2.7.3.1 Immunohistochemistry

Brain sections were incubated for 60 minutes in a blocking solution (10% normal goat serum in phosphate buffer/TritonX-100 solution) before replacing this solution with one that contained the GFP primary Polyclonal Antibody (Thermo Fisher Scientific, catalogue # A-6455) diluted at 1:5000. The TritonX-100 and the normal goat serum was also provided by Professor Cliff Abraham's laboratory. The brain sections remained in this solution for 12 hours in room temperature.

After this period of incubation, the primary antibody was washed out by replacing the blocking solution four times. The blocking solution containing the slices was further replaced with one that contained the secondary Anti-rabbit IgG (H+L) antibody, F(ab')2

Fragment (Alexa Fluor® 555 Conjugate) (CST, catalogue no. # 4413) diluted at 1:400 and were incubated for 2 hours at room temperature.

After this period of incubation, the slices were washed out by replacing the solution four times using phosphate buffer solution. Slices were then mounted onto microscope slides and were applied with minimal antifade solution (VECTASHIELD Antifade Mounting Medium) to slow down the florescence bleaching process.

2.7.3.2 Slice visualization

Slice visualizations were conducted using a ZEISS AXIO Scope.A1 microscope at 5X magnification. Only rats that that expressed the marker of viral expression within the NAc core were included in the data analysis.

2.8 Data analysis

2.8.1 Experiment 1

Using the data of the final 20 sequences of lever presses during T1, the proportion of the active lever presses were calculated for each rat in every group. A one-way ANOVA was performed across all groups to test for differences in performance.

In T2, the number of head entries made during CS presentation and the head entries made in the equivalent time during the inter-trial interval (ITI) leading up to CS presentation was recorded. The former value was divided by the sum of the head entries from the CS and the ITI (CS+ ITI) periods for the final (12th) trial of each rat in every group. Unpaired t-tests were conducted to identify whether group 3 and 4 differed from group 1 in their head entry proportions by the end of T2.

During T1-P, the proportion of presses on the previously active lever was calculated for the first 20 lever presses for each rat in every group. Two-tailed t-tests were performed to test whether groups 2, 3 and 4 differed from group 1.

2.8.2 Experiment 2

A two way ANOVA was used in experiment 2 to test for performance differences during the final 20 trials of T1, last trial of T2 and the first 20 trials during T1-P

3 Results

3.1 Experiment 1

Performance was analyzed from late in T1 (last 20 lever presses) and T2 (final trial) as we were interested in the final level of learning achieved in these tasks. Conversely, during T1-P, the proportion of presses on the previously active lever was analyzed early in the session (first 20 lever presses). The analysis was confined to the first 20 lever presses as the probe session (T1-P) is under an extinction schedule. Therefore, it was reasoned that limiting the analysis to the early responses also limited the likelihood of including the effect of extinction playing a role in the analysis.

3.1.1 T1 analysis

Figure 15 shows performance during the final 20 lever presses of T1 for all groups of rats. The proportion of active lever presses was high in all groups, and an ANOVA showed no significant difference across groups (F (3, 17) = 0.710, P > 0.558). This result indicated that all groups had learned T1 to an equivalent level by the end of training.

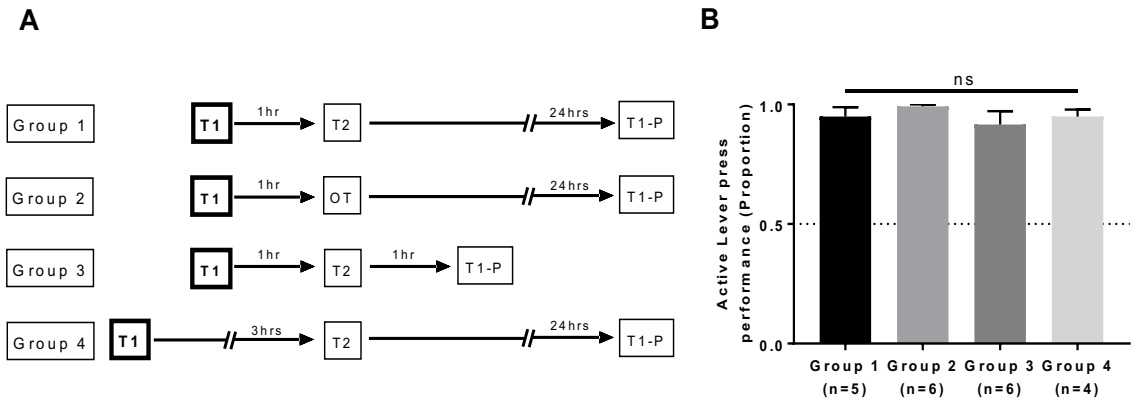

Figure 15: Final 20 lever press performance during T1 across groups. (A) Schematic diagram of experiment 1 highlighting the task (T1) under analysis. (B) Mean and standard error of mean (SEM) for the proportion of active lever presses by the end of T1 (last 20 lever presses) for rats assigned to different groups.

3.1.2 T2 analysis

The mean head-entry performances during the final trial of T2 were also not statistically different between groups 1 and 3 t (9) = 1.87, P = 0.0943 and between groups 1 and 4 t (9) = 0.6154, P = 0.5577 as shown in Figure 16B. This suggested that the different groups of rats that underwent the interpolated T2 condition had a similar learning experience and that any difference in their performances during the following probe condition (T1-P) was due to our manipulated variables (time between sessions).

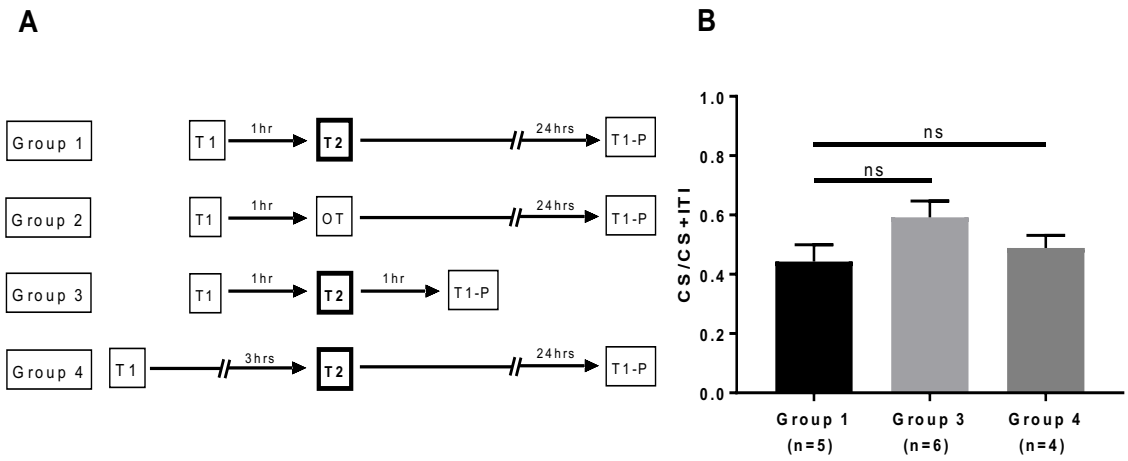

Figure 16: Head entry performance during T2. *(*A) Schematic diagram of experiment 1 highlighting the task (T2) under analysis. (B) Mean and SEM of the ratio of the head entries made during CS presentation and the combined duration of both the CS and the equivalent time leading up to the CS presentation (CS+ITI) in the final (12th) trial of T2.

3.1.3 T1-P analysis

Figure 17A shows that of the first 20 lever presses made during the probe condition (T1-P), rats made a greater proportion of presses on the previously active lever in group 2 on average, compared with group 1. The two tailed t-tests between groups 1 and 2 revealed that the difference in mean is statistically significant (t (9) = -3.096, P = 0.013).

This suggested that the group of rats undergoing the interpolated S-O learning experience performed more poorly in discriminating the previously active lever compared to

the inactive lever than the group that did not experience the interpolated learning experience.

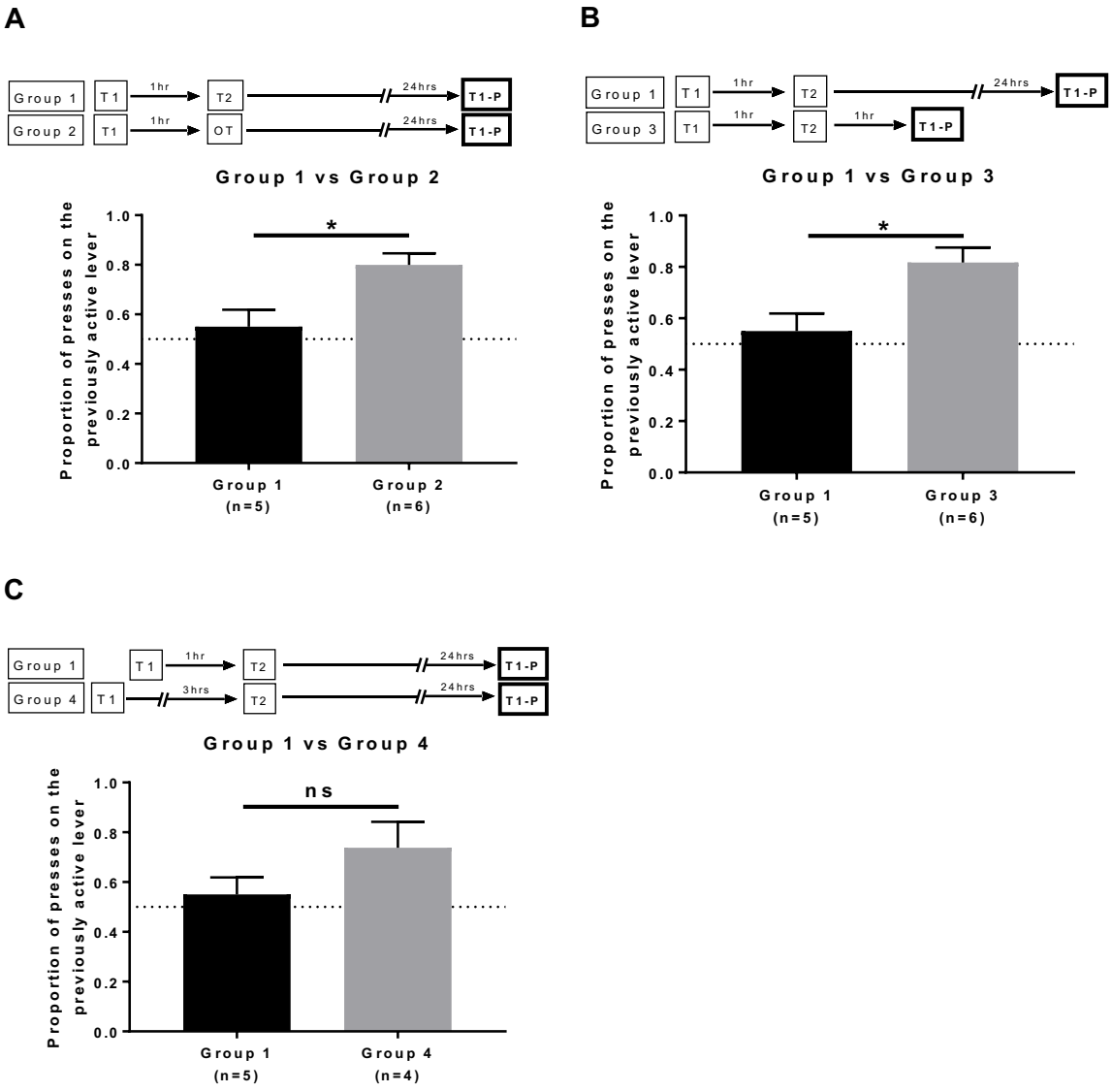

Figure 17: Comparisons of presses on the previously active lever during T1-P. Between-group comparisons of lever press performance early in the T1-P session. (A) Group 1 vs Group 2. (B) Group 1 vs group 3, (C) Group 1 vs group 4. Bar graphs and error bars depict the Mean and SEM of the previously active lever presses made by rats in each group. Schematic diagrams provided above the graphs highlighting the tasks (T1-P) under analysis.

Figure 17B shows that rats also made a higher proportion of presses on the previously reinforced lever when subjected to T1-P one hour (group 3) and not 24 hours (group 1) after the interpolated (T2) learning experience. The t-test between groups 1 and 3 were also statistically significant (t (9) = -2.967, P = 0.016).

While the group of rats (group 4) with the extended time interval between T1 and T2 had a greater proportion of presses on the previously active lever compared to group 1 as seen in Figure 17C, the difference between these means were statistically insignificant (t (7) = -1.553, P = 0.164).

Results in experiment 1 showed that the temporal parameters used in group 1 was most effective in producing an effect of RI. Therefore, these temporal parameters were used in experiment 2 to test whether neural silencing in the accumbens core could alleviate the effect of RI.

3.2 Experiment 2

Viral expression was centered in the NAc core, with some spreading to the dorsal striatum. Figure 18 shows the spread of viral expression schematically and provides a representative photograph of the florescent protein expression in the NAc core.

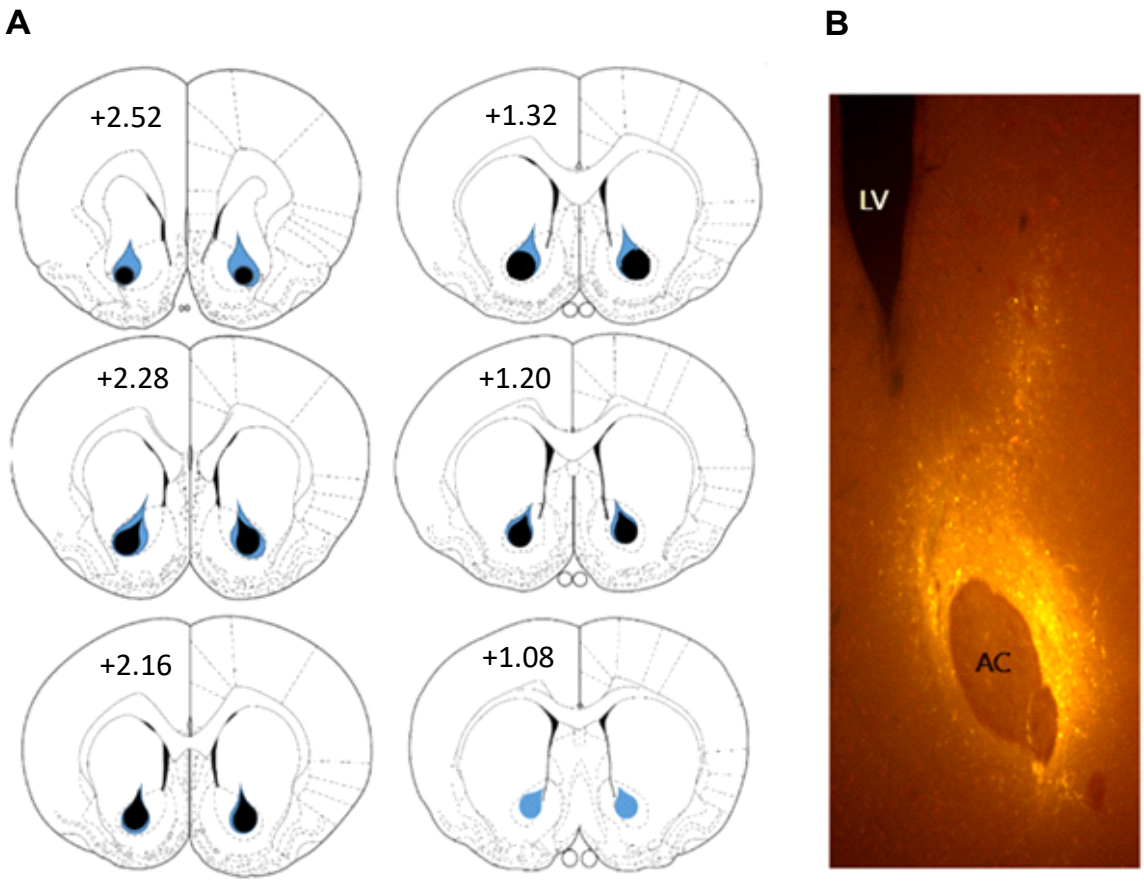

Figure 18: Fluorescent marker of viral expression. (A) Location and spread (where blue and black indicate the maximum and minimum spread of the virus expression in the coronal slice) of the viral expression in the NAc core region as shown on figures adapted from Paxinos et al. (1998). (B) Typical viral expression within the NAc core region as visualized under the microscope, annotated landmarks are the anterior commissure (AC) and the lateral ventricle (LV).

3.2.1 T1 analysis

Figure 19B shows performance during the final 20 lever presses of T1 for all groups of rats. The proportion of active lever presses was high in all groups, and a two-way ANOVA showed no significant difference between viral injection (hM4Di/ GFP) groups (f (1, 15) =

0.567, *P* = 0.463)), no effect of prospective IP injection (*f* (1, 15) < 0.107, *P* = 0.748) and no interaction between the two viral injections and the prospective ligand injections (*f* (1, 15) = 0.184, *P* = 0.674).

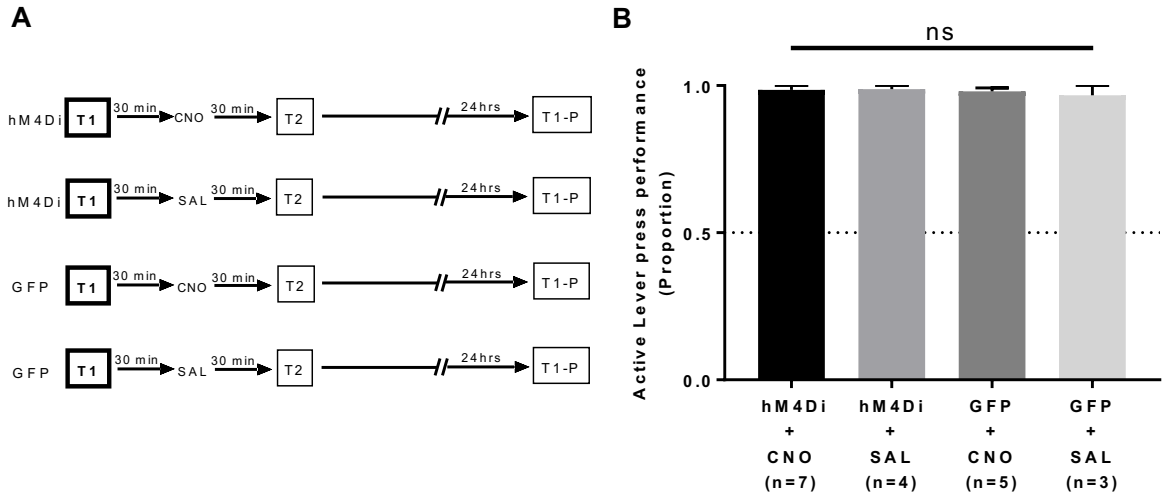

Figure 19: Final 20 lever press performance during T1 across groups. (A) Schematic diagram of experiment 2. (B) Shows the active lever press performance of rats by the end of task 1, bar graphs and corresponding error bars represent the mean and SEM of the proportion of active lever presses.

3.2.2 T2 analysis

Similarly, rats from all four groups performed to a comparable level during the final (12th) trial of the T2 session (Figure 20B). This ratio of head entries was not statistically different across groups with different viral (hM4Di/GFP) injections (*f* (1, 15) = 0.851, *P* = 0.371), or with different IP (CNO/ Saline) injection (*f* (1, 15) = 0.394, *P* = 0.540) and there was no significant interaction between both virus and IP injection (*f* (1, 15) = 2.939, *P* = 0.107). Rats were therefore not different in their performance during both T1 and T2 and any differences found in the following T1-P analysis is unlikely to be due to differences in learning of either T1 or T2.

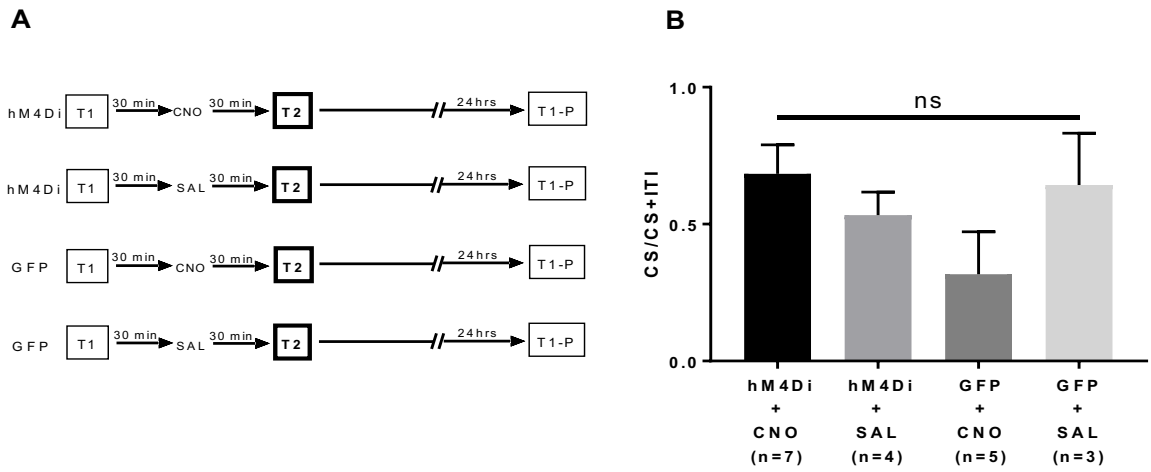

Figure 20: Head entry performance across groups during T2. (A) Schematic diagram of experiment 2. (B) Mean and SEM of the ratio of the head entries made during CS presentation and the combined duration of both the CS and the equivalent time leading up to the CS presentation (CS+ITI) in the final (12th) trial of T2.

3.2.3 T1-P analysis

Figure 21B shows the performance of rats from the experimental group (group 1) and the three control groups (groups 2, 3 and 4). Rats showed no differential effect of viral injection (f (1, 15) = 0.800, P = 0.385), or an effect of the IP injection (f (1, 15) = 0.502, P = .490) and no interaction between the IP and the virus injection. If the experimental group of DREADD indeed had a differential effect to the control condition, there would have been an effect of IP and viral injection. Therefore, the hypothesized result was not seen here.

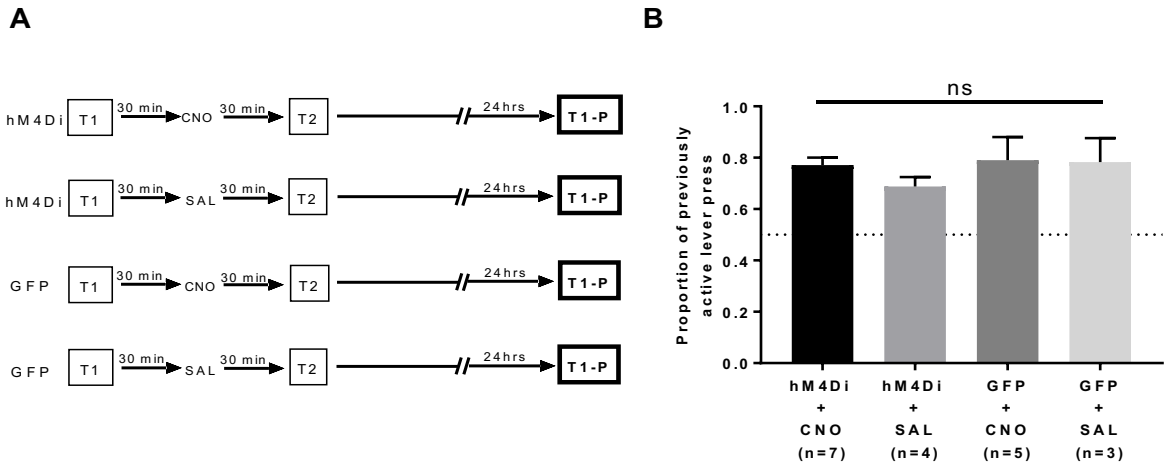

Figure 21: Comparison of presses on the previously active lever across groups during T1-P. (A) Schematic diagram of experiment 2 highlighting the task (T1-P) under analysis. (B) Shows the previously active lever press performance of rats early in T1-P (first 20 lever presses), bar graphs and corresponding error bars represent the mean and SEM of the proportion of previously active lever presses.

4 Discussion

Given the importance of the hippocampus for memory storage in humans, neuroscientific studies on retroactive interference have heavily focused on hippocampal-dependent tasks in rodents. Typically, rats are subjected to a behavioural paradigm that involves learning some form of contextual memory followed by a very similar but behaviourally conflicting interpolated learning experience. When tested for the retention of the initially learnt task at a later time, there is often an impaired level of performance when compared to a control group that did not undergo the interfering learning experience (Butterley et al., 2012; Martinez et al., 2014). However, this effect is not seen if rats are subjected to non-conflicting interpolated learning experiences (Beatty & Shavalia, 1980; Jarrard & Elmes, 1982). Furthermore, silencing the neural activity in the hippocampus during the interpolated learning experience can alleviate the effect of the RI (Martinez et al., 2014).

The current study therefore used the protocol established to both produce and alleviate the effect of RI in hippocampal-dependent learning experiences that are mainly involving context dependent memories and applied it to an appetitive learning situation that does not primarily require the use of the hippocampus (Corbit & Balleine, 2000). Rats were subjected to a paradigm that induced RI using two behaviourally non-conflicting appetitive learning experiences that are both critically reliant on the NAc core (Balleine & Killkross, 1994; Blaiss & Janak, 2009; Hernandez et al., 2002; Kelley et al., 1997; Parkinson et al., 1999). Once a methodological approach that produced RI was found, we tested whether silencing the neural activity of the NAc core when rats underwent the interfering learning experience could also alleviate the effect of RI as seen in the hippocampal study discussed above (Martinez et al., 2014).

4.1 Experiment 1

The first experiment aimed to investigate whether learning a stimulus-outcome (S-O) association can retroactively interfere with a recently learnt action-outcome (A-O) association. This was done by comparing a group of rats that underwent the interfering condition to those that did not.

4.1.1 Group 1 vs Group 2

Using temporal parameters based on those established previously (Martinez et al., 2014), a group of rats (group 1) were first subjected to an operant discrimination procedure (T1) and were then subjected to the classical conditioning procedure (T2) after an hour of hiatus. The performance in the operant discrimination task was tested under extinction (T1-P) 24 hours after experiencing T2. The amount of presses on the previously reinforced lever during T1-P was reduced compared to a group of rats (group 2) that were not subjected to T2. This difference in performance during T1-P shows that undergoing the S-O (T2) learning experience had indeed retroactively interfered with the A-O association (T1). To the best of our knowledge, this is the first study to show that a classical conditioning learning experience can retroactively interfere with the retention of a recently learnt operant discrimination association.

Experiment 1 further aimed to characterize the nature of the effect of RI seen earlier. To do this, different groups of rats (group 3 and group 4) were subjected to varying temporal distances either between the two learning experiences (T1 & T2) or the time between the interpolated learning experience (T2) and the probe session (T1-P). The performance of these groups of rats (group 3 and group 4) during T1-P were compared to that of group 1. The following sections will elaborate on these comparisons and findings.

4.1.2 Group 1 vs Group 3

Previous reports have suggested that RI is more prominent when the retention interval of the learning experiences is elongated (Jarrard & Elmes, 1982; Martinez et al., 2014). Therefore rats in group 3 were subjected to the probe condition (T1-P) one hour after undergoing T2 while maintaining an hour gap between being subjected to T1 and T2. When the performance during T1-P was compared with those in group 1, it was found that the rats in group 3 pressed the previously reinforced lever more than group 1. Therefore, the effect of RI seen in group 1 was diminished when the time lag between T2 and T1-P was reduced. When Martinez et al. (2014) found this effect in their contextual memory paradigm, they speculated that the interpolated learning experience did not affect the short-term memory of their initial learning experience but affected the consolidation of the short-term memory into long-term memory. Therefore, this may also be the same mechanism behind the effect seen in the current experiment. This finding can also be explained using a theory of RI called the temporal distinctiveness theory (Ecker, Brown & Lewandowsky, 2015). The temporal distinctiveness theory suggests that the more isolated the two learning experiences (T1 and T2) are relative to the time of recollection (T1-P), the greater the RI. Since the relative time of recollection (T1-P) in this group is not as isolated from T1 and T2 as in group 1, the findings support both the temporal distinctiveness and consolidation theories. A behavioural test is proposed later in the discussion to tease apart the nature of the RI seen in this paradigm.

Although a difference in the performance during T1-P was observed when the time distance between the T2 and T1-R was reduced, it was important to rule out the possibility that that rats had a differential level of learning T1 and/ or T2 across groups. When tested for this, there were no detectable differences in the learning of the two tasks between the

groups. Thus, the effect seen during T1-P is likely due to the decrease in time lag between T2 and T1-P.

4.1.3 Group 1 vs Group 4

Another test was carried out to assess whether the effect of RI was on consolidation of the learnt A-O association. Rats from Group 4 were subjected to both T1 and T2 but the temporal window between experiencing T1 and T2 was elongated to 3 hours while the time window between T2 and T1-P remained 24 hours. Since earlier studies had found that elongating the temporal window between the initial and interpolated learning experiences alleviated the effect of RI (Martinez et al., 2014), it was hypothesized that rats in group 4 would not show an effect of RI seen in group 1. While rats in group 4 did press the previously active lever more than group 1 on average, this difference was not statistically significant. Again, there were no differences in the level of learning of both T1 and T2 across the two groups, thus supporting the idea that the results were not influenced by differing learning experiences during T1 and T2. Taken together, these results indicate that increasing the temporal window between the two initial learning experiences show a trend towards diminishing the effect of RI

4.2 Experiment 2

Since evidence was found that an S-O learning experience had indeed interfered with a recently learnt A-O association, it was further hypothesized that the effect of RI was due to the neural activity within the NAc core involved with the learning the S-O association. Experiment 2 was therefore carried out to test this hypothesis. Rats were subjected to DREADD mediated neuronal silencing protocol when undergoing the S-O learning experience. When the experimental group and the control groups were compared, rats

showed no behavioural effects that implied an alleviation of RI. This lack of effect could also not be explained by differences in learning experience during T1 and T2 across all groups.

This result was somewhat surprising since Martinez et al. (2014) found an alleviation of RI in their paradigm when silencing the hippocampus during the interpolated learning experience. However, there are potential candidate explanations that might have led to this null finding.

Firstly, the type of neural silencing conducted in this experiment is different than what was used in the contextual memory studies discussed above (Martinez et al., 2014). Martinez et al. (2014) silenced the neural activity of excitatory neurons by applying muscimol, a GABA-A receptor agonist. The level of neural silencing achieved by muscimol is much stronger than that achieved using DREADD (Smith et al., 2016). For example, Chang, Todd, Bucci, and Smith (2015) recorded the activity of neurons in the ventral palladium that expressed hM4Di on their cell surface. They found that activity of the hM4Di expressing cells only underwent a 60% decrease compared to their pre-CNO baseline measurement. This was further supported by a study that found that the normal calcium spikes that were related to increases in neuronal activity was dampened but not completely diminished with DREADD based neural silencing (Cichon and Gan, 2015). Although chemogenetically silencing neural activity in the accumbens have had a behavioural impact in other paradigms (Cassataro et al., 2013), it might not have been sufficient to produce behaviourally-relevant effects in the current study. Therefore it might be worthwhile to test whether a more robust silencing of the NAc core during the interpolated (T2) learning experience would alleviate the RI seen in this paradigm. In support of this proposal, a study conducted by Blaiss and Janak (2009) showed that intra accumbens infusion of a combination of both baclofen and muscimol, which are both GABA-B and GABA-A receptor agonists respectively prevented the

expression of an already learnt pavlovian association. Since it can be argued that learning of an S-O association is the result of repeated, correct classical conditioning responses across trials in a session (such as T2), it is possible that pharmacological inactivation of the accumbens core could have been better suited in the current paradigm to prevent learning. The result from the analysis of the performances of T2 also supports the need for a stronger silencing procedure. The analysis showed no performance differences between the experimental group and the control groups in experiment 2. If neural silencing was indeed sufficient in preventing the learning of the S-O association during T2, rats may have shown a poorer level of performance by the end of T2 in the experimental group compared to the control groups. Future studies could better assess the relation between suppression of NAc activity, learning of an S-O association, and subsequent RI.

Although there were no differences in the performance between groups during T1-P in experiment 2, visual examination of the results showed a greater proportion of lever presses in general across all conditions in experiment 2 compared with group 1 of experiment 1. This suggests that rats in experiment 2 might not have had a strong enough effect of RI as those in experiment 1. This result raises the possibility that strengthening the effect of RI might be achieved by either decreasing the level of learning during T1 or increasing the level of learning during T2.

Rats were subjected to T1 for a 50-minute duration, during this time, rats were continuously reinforced for pressing one lever and were not reinforced when pressing the other lever. These rats might have been able to learn the operant discrimination long before the elapsed 50-minute duration leading to overlearning by the termination of the T1 session. Evidence has also shown that overlearning has reduced the effect of RI in humans (McAllister, 1952). Therefore, one way to prevent the overlearning is by limiting the amount

of exposure to the T1 session. To do this, the session could either be decreased in duration (e.g. 40 or 30 minutes in duration) or the session could be scheduled to end when a certain criteria for the amount of consecutive reinforced lever presses are made (e.g. 10 or 20 consecutive lever presses on the reinforced lever). Additionally, rats can undergo a more comprehensive learning experience during T2 in order to increase the activity of the nucleus accumbens that is thought to interfere with the consolidation of T1. In the current paradigm, rats undergo only 12 consecutive trials during the T2 session, therefore, doubling or tripling the amount of trials experienced by rats could increase the likelihood of T2 interfering with T1.

4.3 General discussion

So far, the results from both experiments have been interpreted in terms of disruption of memory consolidation of T1 by T2. However, as mentioned earlier, a competing cognitive theory may also explain these findings. The temporal distinctiveness theory suggests that the ratio of the time interval between the two learning experiences (the initial to the interpolated learning experience) and the time interval from the interpolated learning experience to the test (probe) session could be the main contributor of the effect of RI (Brown, Neath, & Chater, 2007). According to the temporal distinctiveness theory, the temporal context in which an event takes place becomes encoded in the memory. Thus, if two new learning experiences take place in close proximity the effect of RI would be greater when tested after a longer period of time than if tested after a shorter period of time. As time passes further from the two initial learning experiences, the resolution between the two learning experiences become poorer, thus resulting in the memories of the two learning experiences competing during retrieval. Therefore temporal distinctiveness theory suggests that the more isolated the two learning experiences are in relation to the time of recall, the

greater the interference. In this view, it is not aspects of consolidation that is the cause of RI, but the temporal distinctiveness of the learning experiences at recall.

Ecker et al. (2015) tested whether temporal distinctiveness theory could explain RI more than the consolidation theory in a human verbal learning paradigm. In this study, participants were presented with two lists (L1 and L2) of 10 words and were tested (T) for the retention of one of the two list of words at a later time. However only the retention of list 1 was analyzed and compared across groups.

As shown in Figure 22, there were four groups with varying differences in time gaps between the presentation of the two lists and the time between the second list and the test time. One time duration was 60 seconds (short) and the other was 240 seconds (long) and four different groups were subjected to different combinations of the time gaps between the three conditions. The first group (SS) had a short duration between both the two list learning conditions and between the second list and the test condition, the second group (SL) had a short duration and a long duration while the third group (LS) experienced a long duration and a short duration and the last group (LL) experienced long durations between all three conditions.

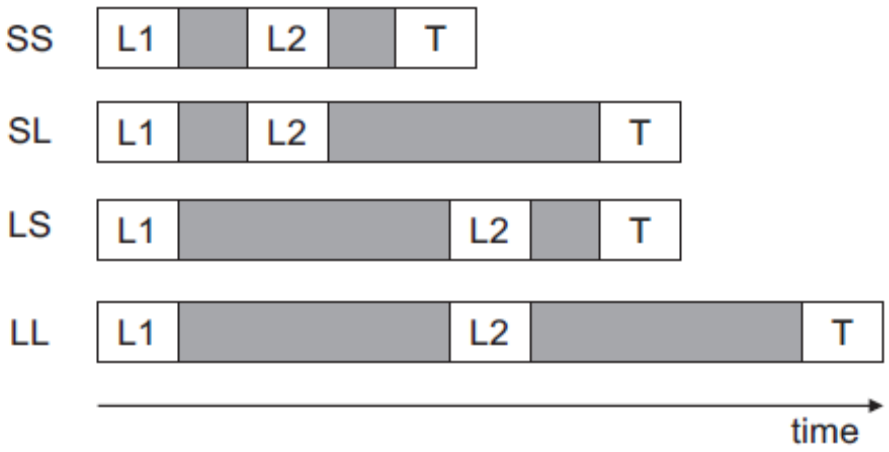

Figure 22: Adapted figure from Ecker et al. (2015) depicting the temporal parameters underwent by participants between each condition. Participants either experienced a short interval (S) that lasted 60 seconds or a long interval (L) that lasted 240 seconds.

It was predicted that if retroactive interference strictly followed the predictions of consolidation theory, performance would be equal between SL and SS but be worse than both LL and LS while there would be no difference between LL and LS (i.e., SL = SS < LL = LS). Alternatively, if temporal isolation of the learning experiences (T1 and T2) relative to the recall period (T) is mostly important, the performance during test session would be equal between SS and LL while SL would yield the least performance in retention and LS would produce the best performance in retention (i.e., SL = SS < LL = LS).

Results from their analysis were consistent with the latter hypothesis and that the effect of RI was better explained by the temporal distinctiveness theory. This theory also emphasizes that the effect of RI was on the retrieval during test session rather than the disruption of the storage of the initially learnt experience. Ecker et al. (2015) further mentioned a lack of testing the validity of the temporal distinctiveness theory in the animal literature, making the current paradigm a good candidate to test this theory.

A modified protocol developed by Ecker et al. (2015), could be administered in the current paradigm as shown in Figure 23. Rats can similarly undergo differential temporal

distances between the two learning sessions (T1 and T2) and between the interpolated (T2) learning experience and the probe session (T1-P). According to Ecker et al. (2015), results from experiment 1 already shows a violation of consolidation theory since rats from group 3 (SS) fared better than group 1 (SL). However, a similar finding by Martinez et al. (2014), explained this effect as RI on the consolidation of a short term memory into a long term memory and not on the short term memory itself. Therefore, any differences in the proposed LS and LL conditions will be able to reveal whether the current paradigm supports the temporal distinctiveness theory or the consolidation theory. If the performance during T1-P does not differ between LS and LL conditions, it can be suggested that T2 interferes with the consolidation of T1 as both conditions undergo the interpolated learning experience much longer following the average expected consolidation time window. Alternatively, if performance of T1-P is better by rats in the LS condition than the LL condition, it maybe because the two learning experiences are more isolated for rats in the LS condition in relation to the LL condition. This would support the temporal distinctiveness theory as the causative reason behind the RI experienced by these rats.

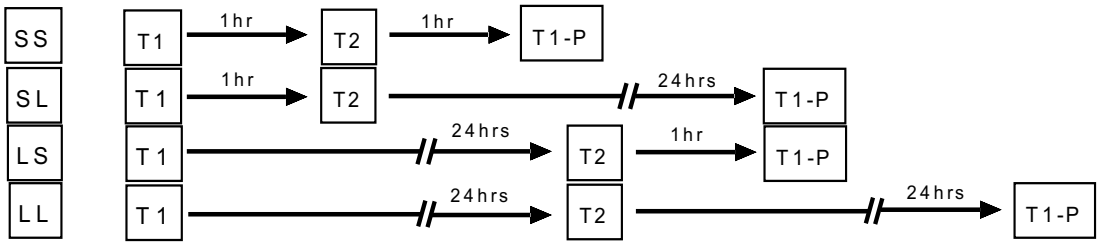

Figure 23: A modified protocol used by Ecker et al. (2015) to test the current paradigm.

4.4 Final remarks and implications

In conclusion, the current study showed that being involved in learning a classical conditioning task has an effect of RI on the retention of a recently learnt operant discrimination task. To the best of our knowledge, this was the first study to show this effect.

We also made an initial effort to extend our study to investigate the neural underpinnings of the current paradigm, and our results point the way to future areas of inquiry. We suggest that further optimization of our paradigm could pave the way towards exploration of further avenues that are largely absent from rodent RI studies. One possible strategy for future research is testing whether the RI seen in this paradigm is due to retrieval (due to temporal distinctiveness) or due to prevention of storage (consolidation) of the initial learning experience.

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