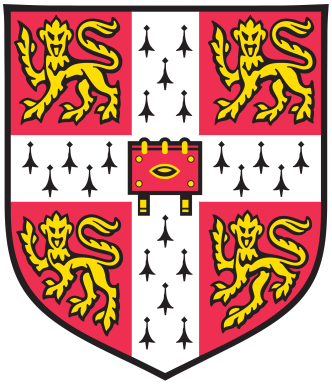
An assessment of reconsolidation blockade to disrupt memories relevant to psychiatric disorders

A dissertation submitted for the degree of

Doctor of Philosophy



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Contents

Preface

The following work was conducted with the Department of Psychology, University of Cambridge during the years of 2013-2016 under the supervision of Doctor Amy L Milton.

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit for the relevant Degree Committee.

Acknowledgements

I would firstly like to thank Doctor Amy Milton for supervision, guidance and encouragement during the course of my PhD. I am also very grateful for the support received throughout from my adviser Professor Barry Everitt. This thesis has been greatly improved as a result of the input of both these individuals.

During the course of my PhD I have been humbled by the willingness of those around to me to offer support and guidance. I am particularly grateful to Doctor Gonzalo Urcelay who was a source of great inspiration particularly in the early stages of this PhD. I would also like to thank Chiara Guiliano for her continued willingness to discuss the finer details of second-order schedules of reinforcement, Yolanda Peña-Oliver and Emiliano Merlo for instruction in surgical techniques and both of these individuals, alongside Doctor Emma Cahill for numerous discussions of the promises and pitfalls of academia, science and reconsolidation research. I also thankful for the support of Rudolf Cardinal, Mickaël Puaud, David Theobold, Colin McKenzie, Omar Perez, Richard Fieldsend, Rebecca Hubble, Hania Wyciszczok, Anthony Dickinson and the staff in the Combined Animal Facility.

I would also like to thank all of those that have provided support on a personal level during the course of my PhD. It wouldn't be true to say that there hasn't been a dull moment in Room 209, but my fellow doctoral students have made it a much more enjoyable environment to write my thesis in. I am particularly grateful to Zara Goozée who has provided support throughout and is responsible for the vast majority of cards on my desk. I am thankful to all my friends and family that have supported me, including Ryan Hamnett, Rudi Carter and the members of Magdalene MCR.

Publications

Work from this thesis has been presented in the following meetings and journal articles.

Conference abstracts

1. Vousden, G.H. and Milton, A.L. (2014). Exploring the goal-directedness of context-induced renewal. *Poster presented at Cambridge Neuroscience Seminar 2014 – Brain Science and Mental Health*, 14 March 2014; Cambridge, UK.

2. Vousden, G.H. and Milton, A.L. (2015). Investigations in context-induced renewal: effects of overtraining and NMDA receptor antagonism at memory retrieval. *Poster presented at BNA2015: Festival of Neuroscience*, 12-15 April 2015; Edinburgh, UK.

3. Vousden, G.H. and Milton, A.L. (2015). Investigations in context-induced renewal: effects of overtraining and NMDA receptor antagonism at memory retrieval. *Poster presented at Cambridge Memory Meeting 2015*, 27 April, 2015; Cambridge, UK.

4. Vousden, G.H., Hubble, R.J., Peña-Oliver, Y., Everitt, B.J., Milton A.L. (2015). What makes a cocaine-associated memory reconsolidate? *Poster presented at European Brain and Behaviour Society & European Behavioural Pharmacology Society Joint Meeting*, 12-15 September 2015; Verona, Italy.

5. Vousden, G.H., Hubble, R.J., Peña-Oliver, Y., Everitt, B.J., Milton A.L. (2016). What makes a cocaine-associated memory reconsolidate? *Poster presented at Cambridge Neuroscience Seminar 2016 – New Directions*, 17 March 2016; Cambridge, UK.

Papers

1. Vousden, G.H. and Milton, A.L. (2017). The chains of habits: too strong to be broken by reconsolidation blockade? *Current Opinion in Behavioral Sciences*, **13**: 158-163.

List of acronyms

AMPA Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ANR Acquisition of a new response

ANOVA Analysis of variance

A-O Action-outcome

ASO Antisense oligodeoxynucleotide

A-P Anterior-posterior

BLA Basolateral amygdala

β-lac Clasto-Lactacystin-β-lactone

BDNF Brain-derived neurotrophic factor

CS Conditioned stimulus

CEN Central nucleus of the amygdala

CIR Context-induced renewal

CPP ($\pm$)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid

CTA Conditioned taste aversion

DCS D-cyloserine

DLS Dorsolateral striatum

DMS Dorsomedial striatum

DSM Diagnostic and statistical manual of mental disorders

ECS Electro-convulsive shock

EE Environmental enrichment

ERK Extracellular signal-regulated kinase

FI Fixed interval

FR Fixed ratio

G-G Greenhouse-Geisser

GABA Gamma-aminobutyric acid

H-F Huynh-Feldt

IEG Immediate early gene

IL Infralimbic

ip Intraperitoneally

im Intramuscular

ISI Inter-shock interval

K-S Kolmogorov-Smirnov

LiCl Lithium chloride

LTD Long-term depression

LTP Long-term potentiation

LTM Long-term memory

LTMT Long-term memory test

M-L Medial-lateral

MK-801 (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate

MAPK Mitogen-activated protein kinase

mTOR Mammalian target of rapamycin

mGluR Metabotropic glutamate receptor

ml Millilitre

mRNA Messenger ribonucleic acid

NMDA N-methyl-D-aspartate

ODN Oligodeoxynucleotide

PBS Phosphate buffered saline

PFA Paraformaldehyde

PIT Pavlovian-instrumental transfer

PKA Protein kinase A

PL Prelimbic

PR-LTMT Post-reactivation long-term memory test

PTSD Post-traumatic stress disorder

PE Prediction error

RPM Responses per minute

s Seconds

S-R Stimulus-response

sc Subcutaneous

STM Short-term memory

VI Variable interval

VR Variable ratio

VT Variable time

VTA Ventral tegmental area

US Unconditioned stimulus

Summary

Consolidated memories can become reactivated in order to permit the integration of new information into the memory trace. Blockade of the resultant process, reconsolidation, with NMDA receptor antagonists or protein synthesis inhibition can lead to a decrease in subsequent memory expression. This may offer a potential tool for the treatment of psychiatric disorders characterised by maladaptive memories, including drug addiction and post-traumatic disorder.

Given the importance of instrumental associations in supporting drug addiction experiments in Chapters 3 & 4 aimed to disrupt reconsolidation of these memories. Treatment with an NMDA receptor antagonist prior to retrieval sessions of various durations was not able to consistently prevent reconsolidation of these associations.

Drug addiction is characterised by memories that have been formed not over days or weeks, but months or years. Experiments in Chapters 5 & 6 therefore investigated how the extent of training affects the propensity of an appetitive pavlovian memory to reconsolidate. Experiments in Chapter 5 were not able disrupt reconsolidation of these memories after a relatively short period of training. In Chapter 6 attempts to disrupt reconsolidation of a cocaine-seeking memory having undergone extensive training (>1 month, designed to promote the formation of drug-seeking habits) were also unsuccessful. However, when animals were trained in a similar fashion to respond for a food-reinforcer treatment with a NMDA receptor antagonist prior to a reactivation session resulted in a decrease in food-seeking behaviour the following day. However, this deficit was only found in the first test session; drug treatment had no effect on responding following reminder of the memory.

If data from preclinical studies are to inform future psychiatric treatments the findings from these works must be robust and replicable. Experiments in previous chapters encountered several issues in this regard, namely the repeated inability to prevent reconsolidation with NMDA receptor antagonism. Given that reconsolidation of auditory fear memories is well characterised a final series of experiments in Chapter 7 used this procedure to explore the possible reasons for the fleeting or absent effects of disrupted memory reconsolidation in previous chapters. Despite the use of similar methods as published reports showing decreases in memory expression as a result of blockade of reconsolidation it was not possible to disrupt this process with NMDA receptor antagonism or protein synthesis inhibition. Results suggested that the failure to observe reactivation-dependent amnesia was due to the amnestic agent used not being able to prevent reconsolidation, should it be taking place, and a failure of the given retrieval trial to result in memory reactivation.

On numerous occasions throughout this thesis it was not possible to disrupt memory reconsolidation. One difficulty in interpreting null data of this nature is that it is often unclear whether the results are due to insufficient retrieval conditions to result in memory reconsolidation, or an inability of the pharmacological agent to disrupt this process. The final experiments of this thesis raised the possibility both of these issues may have contributed in tandem towards this inability to prevent memory reconsolidation.

Chapter 1: General introduction

Introduction

Associative memories are formed upon the temporally linked presentation of stimuli or actions and outcomes; an environmental context can become associated with trauma, golden arches with fast-food or the act of smoking with the delivery of nicotine. Whilst the ability to form these associations is evolutionarily advantageous, these memories can become maladaptive and obtrusive, exerting an overwhelming control over an individual's behaviour. Although the principles underlying these memories are simple, the psychological, neuroanatomical and molecular bases are highly complex and far from entirely understood. A better knowledge of these processes may be able to inform future treatments for psychological disorders characterised by memories which are intrusive or maladaptive including post-traumatic stress disorder (PTSD) and drug addiction.

The pharmacological systems and molecular cascades required for two processes, consolidation and reconsolidation to take place will first be discussed. These two processes appear to be essential for the maintenance of memory, since disruption of either can lead to amnesia for a recently formed or retrieved memory, respectively. The function of reconsolidation and psychological requirements for it to take place will then be reviewed, with particular attention paid to the role of prediction error. The ability to disrupt subsequent memory retrieval with the administration of pharmacological agents may be used to treat psychological disorders characterised by maladaptive memories; the potential for the treatment of PTSD and drug addiction will be discussed in this regard. Finally, the potential difficulties that need to be overcome before such interventions are adopted in the clinic will be considered and how this thesis will attempt to address these issues will be outlined.

Memory consolidation and reconsolidation

Memory formation does not occur immediately; a cascade of processes is necessary for the stabilisation of the trace following acquisition, a process termed consolidation. In 1900, Müller and

Pilzecker reported that the recall of nonsense syllables was impaired by the learning of a second set of syllables immediately following the first encoding episode. This suggested memory acquisition was not immediate, but rather a *consolidation* process was occurring after the first learning episode. It was not until almost half a century later when this concept was investigated further, with two papers reporting that administration of electro-convulsive shock (ECS) to rodents immediately following memory acquisition resulted in deficits in subsequent retrieval (Duncan, 1949; Gerard, 1949). Further seminal investigations in this field have demonstrated that whilst blockade of cellular processes such as protein synthesis has no immediate effect on task performance, this treatment results in memory impairments in tests several days later (Agranoff *et al.*, 1966). This latter distinction has lent support to the notion that there are multiple memory systems within the brain, with only long-term memory requiring protein synthesis. Short-term memory (STM) is hypothesised to be the result of electrical activation of synapses (Hebb, 1949) in; it is through the process of consolidation that this fleeting representation is transferred to long-term memory (LTM) (McGaugh, 1966; McGaugh, 2000, see Figure 1.1A). Extensive inactivation studies have demonstrated that whilst the hippocampus is required for storage of recent memories, as these become more remote they are transferred, likely through consolidation, to more cortical regions including the anterior cingulate and prelimbic cortices and the temporal cortex (Wiltgen *et al.*, 2004).

A growing literature suggests that consolidation is not the only period during which a memory can become active and vulnerable to disruption. Studies as early as 1968 complemented previous work demonstrating post-acquisition ECS could result in amnesia; by showing this treatment could similarly induce subsequent deficits in memory expression when applied immediately following memory retrieval (Misanin *et al.*, 1968; Schneider and Sherman, 1968). Furthermore, similar to consolidation, subsequent memory expression is impaired if competing learning takes place following memory retrieval (Gordon, 1977a). This apparent process can also be enhanced with administration of the glycine and acetylcholine receptor antagonist strychnine following memory recall (Gordon, 1977b). These works highlighted the possibility that through the process of retrieval memories could be *re*activated and once again enter a short-term store, triggering a second period in which they could become manipulated. It was not until much later, however, that this idea was explored further, with works in 1997 and 2000 using the term *reconsolidation*, noting its reliance upon mechanisms similar to consolidation (Nader *et al.*, 2000; Przybyslawski and Sara, 1997). Not only do both of these processes require protein synthesis (e.g. Nader *et al.*, 2000; Schafe and LeDoux, 2000), but importantly, disruption of reconsolidation has no effect on STM, only affecting memory expression

>24h following memory retrieval (e.g. Nader *et al.*, 2000). These results, alongside others, have resulted in the now widely accepted belief that once consolidated, memories can become destabilised and reactivated. Once in this active state, the memory is held in a store similar to STM and can return to an inactive state through reconsolidation (see Figure 1.1B).

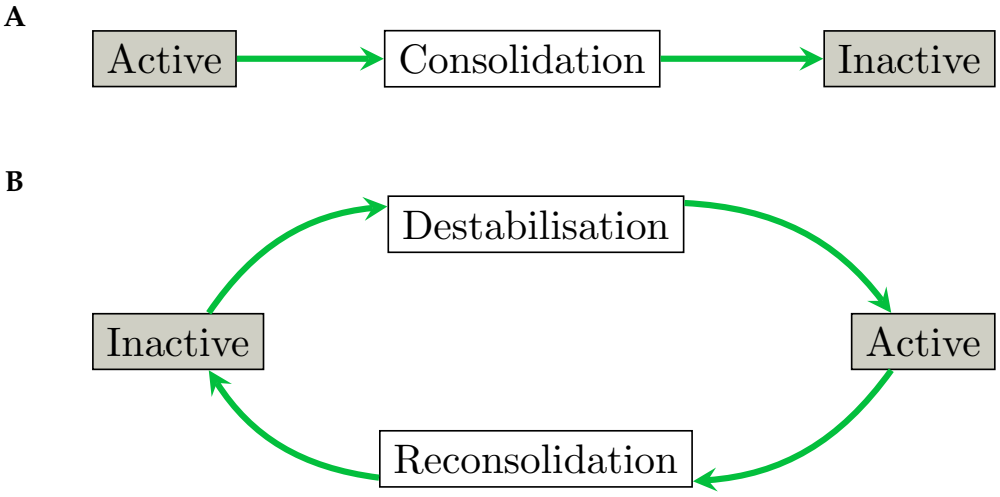


Figure 1.1: Processes involved in the acquisition and maintenance of memory. **A**: Immediately following the formation of a memory the recently formed association is held in a short-term store and is 'active'. This store cannot maintain memory permanently and, through the process of consolidation, the memory is transferred to a long-term store and becomes 'inactive'. **B**: Under certain conditions of retrieval memories can undergo destabilisation and once again enter an active state, held in a similar short-term store. The memory can then be returned to its inactive state through reconsolidation. Disruption of this process leaves the memory in an unstable state and results in subsequent amnesia.

The function of both consolidation, and particularly reconsolidation, has been a topic of great debate. It has been suggested that consolidation allows only those memories that are of adaptive value to be maintained (McGaugh, 1966). Artificial stimulation of stress and arousal systems with corticosterone and adrenaline results in enhancements in memory consolidation (e.g. Gold and Buskirk, 1975; Roozendaal *et al.*, 2006a; Zorawski and Killcross, 2003), raising the possibility that endogenous activation of these systems in response to motivationally significant events ensures they are stored with sufficient strength to ensure appropriate responses to similar events in the future. The function of reconsolidation is less clear, given that it could be considered maladaptive to repeatedly expose memories to interference. It has been suggested that this process may similarly serve to ensure the maintenance of long-term memories; there is evidence that memory reactivation can confer a resistance to decreases in retrieval as time since an event passes (i.e. forgetting). Reconsolidation is also theorised to permit the integration of novel information into existing traces (Lee, 2009; Nader and Einarsson, 2010).

Amongst others, the early research on consolidation and reconsolidation provided a springboard for a wealth of ongoing research into these memory processes. The requirements for consolidation and reconsolidation to take place have been explored through administration of various compounds preventing specific neurochemical processes and molecular cascades. Through careful parametric modulation of the psychological events that result in memory reconsolidation the potential functions of this process have also been explored. Here some of the neurochemical, molecular and psychological processes required for memory consolidation and reconsolidation will be outlined.

Neurochemical basis of memory consolidation and reconsolidation

Numerous neurochemical systems have been implicated in memory consolidation and reconsolidation. Drugs can be administered either prior to, or immediately following training sessions to demonstrate an impairment in consolidation. However, an STM test is required when drugs have been administered before training sessions to ensure any resulting effects are the result of deficits in consolidation, rather than acquisition. Drugs can be administered either before or after reactivation sessions in order to demonstrate impairments in memory reconsolidation. The neurotransmitters addressed below are outlined in a level of detail approximately reflective of their relevance to this thesis. The studies listed here are not exhaustive and nor does this list reflect the only neurotransmitter systems that are required for memory reconsolidation.

Glutamate

The vast majority of research exploring the neurochemical basis of learning and memory to date has focussed on glutamate. This is primarily the result of its integral role in long-term potentiation (LTP), the hypothesised, but much debated, cellular mechanism of memory (e.g. Bliss and Lømo, 1973; Bliss and Collingridge, 1993; Malenka, 1994; Stevens, 1998).

Glutamate binds to two types of receptors: ionotropic, where direct binding of glutamate results in the opening of the channel and metabotropic (mGluR), where the binding of glutamate results in the release of secondary messengers which in turn gate the ion channels. There are three subtypes of ionotropic glutamate receptors, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA) and kainate . There are 8 different mGluR receptor subunits, divided into group I(1,5), II(2,3) and III(4,6,7,8).

NMDA receptors

The opening of NMDA receptors requires not only postsynaptic binding of glutamate, but also membrane depolarisation. Under resting conditions a magnesium block generates a positive charge within the receptor, preventing entry of calcium. Firing of several adjacent axons, alongside release of glutamate from presynaptic axons, removes this magnesium block, permitting influx of calcium into the postsynaptic cell, triggering activation of calcium-dependent kinases (Kandel *et al.*, 2000). The rate of calcium entry typically determines whether there is an increase (LTP) or decrease in synaptic strength (long-term depression (LTD)) (Mulkey and Malenka, 1992). These apparently unique properties allow NMDA receptors to act as coincidence detectors, or "AND" gates, only permitting conduction through the channel when there is simultaneous glutamate in the synaptic cleft and post-synaptic depolarisation (Paoletti and Neyton, 2007).

These characteristics of the NMDA receptor make it well suited to detect relationships between coincident external events. Indeed, these receptors appear to be required for auditory-fear conditioning. In this task, concomitant presentation of a previously neutral stimulus alongside an aversive event (a mild foot-shock; unconditioned stimulus (US)) results in a conditioned response to the once neutral stimulus; it becomes a conditioned stimulus (CS)(see Figure 1.2B). Administration of an NMDA receptor antagonist systemically or into the lateral ventricle prior to acquisition of these pavlovian memories (see Figure 1.2C) results in subsequent impairments in memory expression (Dalton *et al.*, 2012; Kim *et al.*, 1991). Local infusion of these drugs into the basolateral amygdala (BLA), a region strongly associated with memories of this nature (Hitchcock and Davis, 1986), has comparable effects (Miserendino *et al.*, 1990). Similar results have been reported for contextual fear, where a context, rather than a discrete cue is paired with a shock (see Figure 1.2A). Systemic administration of NMDA receptor antagonists prevents learning in this task (Stiedl *et al.*, 2000), as does local infusion of these drugs into the amygdala (Fanselow, 1994) or hippocampus (Matus-Amat *et al.*, 2007), a region implicated in spatial memories (Fanselow, 2000; Logue *et al.*, 1997; McDonald and White, 1994; Sutherland and Rudy, 1989).

Extensive research in the consolidation field has used avoidance tasks, which may be 'active' or 'passive', where animals are trained to move between contexts in order to avoid shock delivery. Whilst active avoidance requires animals to make a response in order to avoid a shock, passive avoidance requires that they do not. Administration of NMDA receptor antagonists either systemically (Mathis *et al.*, 1991; Zajaczkowski *et al.*, 1997) or directly into the hippocampus (Alvarez and Banzan, 1999; Roesler *et al.*, 1998) impairs learning in both types of avoidance memory.

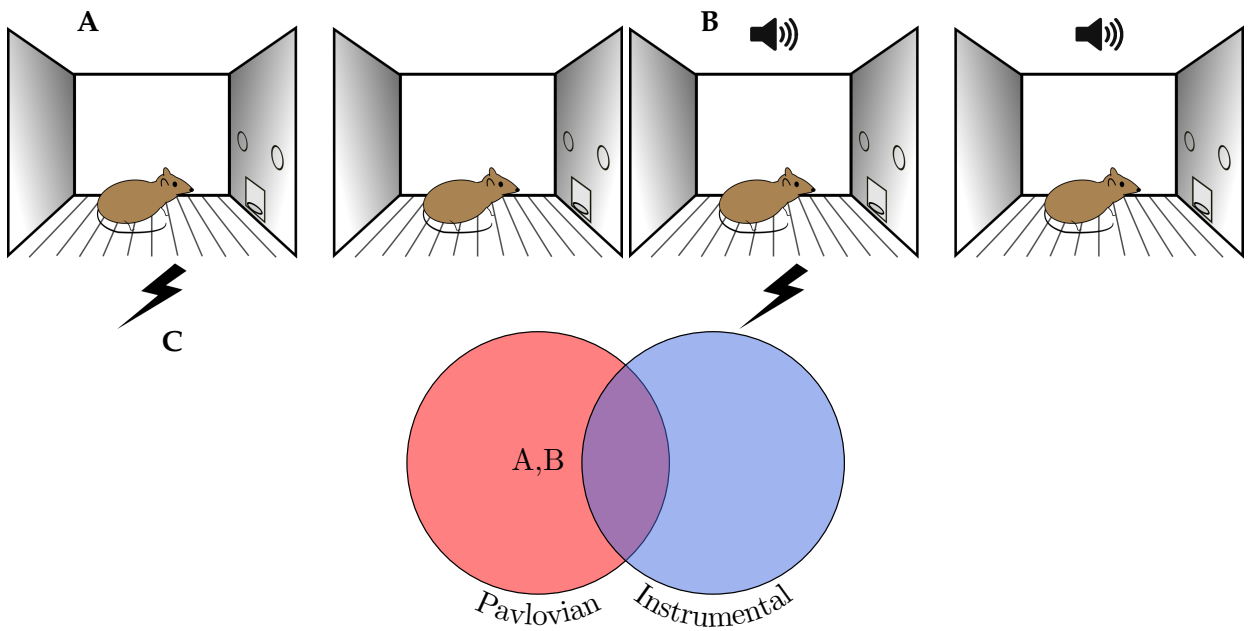


Figure 1.2: Tasks used to study aversive pavlovian memories in rodents. In each case, the left hand box shows the procedure used in training and the right used at test. Lightening symbol represents delivery of foot-shock. **A**: Contextual fear conditioning: exposure to a shock-paired context results in freezing. **B**: Auditory fear conditioning: presentation of a shock-paired auditory CS results in freezing. Speaker symbol represents delivery of auditory stimulus. **C**: Venn diagram showing the suggested memory mechanisms required for performance in these tasks.

It is important to consider whether NMDA receptor antagonists are able to prevent consolidation, or if the memory deficits occurring as a result of administration of these drugs results in an impairment in acquisition. A specific ability to disrupt consolidation can be demonstrated with post-training drug administration or unimpaired STM but impaired LTM. Whilst NMDA receptors do appear to have a specific role in memory consolidation, rather than acquisition, this appears to be task specific. Antagonism of these receptors both systemically and directly within the hippocampus does not affect STM expression, this treatment results in impaired recall of a spatial memory when tested once the association would have otherwise been consolidated (McDonald *et al.*, 2005; Steele and Morris, 1999). NMDA receptor activation is also required for the consolidation of object-recognition (Lima *et al.*, 2005) and odour-reward memories (Tronel and Sara, 2003), effects that could not be attributed to a failure in acquisition. However, post-training intracerebroventricular administration of NMDA receptor antagonists has no effect on subsequent fear expression (Kim *et al.*, 1991) and pre-training systemic or intra-amygdala administration of these drugs results in both short and long-term deficits in memory expression (Rodrigues *et al.*, 2001). These findings suggest that whilst NMDA receptor activation is required for *acquisition* of fear memories, there is less evidence for a *consolidation* specific role of these receptors for these memories.

The formation of associations with stimuli and appetitive outcomes requires NMDA receptor activation, with antagonism of these receptors preventing morphine (Couto *et al.*, 2004; Tzschentke and Schmidt, 1995) and cocaine (Cervo and Samanin, 1995) induced conditioned-place preference. In this task animals are repeatedly treated with a drug and subsequently confined to a salient environmental context. Preference for the drug-paired compartment in comparison to a neutral context is taken as an index of memory. It is unlikely that NMDA receptors are only involved in the reinforcing properties of these drugs, or that they are only required for the acquisition, but not consolidation of conditioned-place preference, since post-training administration of NMDA receptor antagonists similarly results in impairments in learning (Alaghband and Marshall, 2013; Tomazi *et al.*, 2016). However, there are few, if any, reports of unimpaired STM, but not LTM deficits occurring as a result of NMDA receptor antagonism in these reward-associated memories.

NMDA receptors are also involved in the reconsolidation of conditioned-place preference memories. NMDA receptor antagonism prior to retrieval of cocaine (Brown *et al.*, 2008) and amphetamine (Sadler *et al.*, 2007) conditioned-place preference associations results in deficits in subsequent expression of these memories. However, the use of conditioned-place preference tasks suffers from similar issues to classic active and passive avoidance tasks, in that it is unclear whether performance in these tasks is relies upon pavlovian associations between the context and the reinforcer, or whether the animal is superstitiously spending more time in the drug-paired compartment in an attempt to receive further reinforcement.

Through the use of more sophisticated behavioural tools it has been shown that NMDA receptors are required for reconsolidation of pavlovian appetitive memories. The autoshaping procedure has been used to specifically investigate reconsolidation of pavlovian memories. Typically, food-delivery is paired with illumination of a CS light and presentation of a lever beneath it (although this procedure it not always used and may not necessarily be optimal, see Bussey *et al.*, 1997). Over time, animals will approach the light CS and happen to depress the lever acting as a measure of approach. Animals may also press and bite on the lever, despite these responses being without consequence (see Figure 1.3A). Reconsolidation (and consolidation; Dalley *et al.*, 2005) of these memories has been shown to rely on NMDA receptor activation (Lee and Everitt, 2008c). Appetitive pavlovian associations can also be probed through the assessment of the ability of a reward-paired cue to promote acquisition of a new response (ANR); the rewarding properties of this stimulus are assessed through the ability of contingent CS delivery to maintain responding of an otherwise

non-rewarded instrumental response (see Figure 1.3B). Systemic (Lee and Everitt, 2008a) or intra-BLA (Milton *et al.*, 2008a) NMDA receptor antagonism can prevent reconsolidation of the pavlovian memories underlying this responding.

Instrumental tasks can also be used to study interaction between pavlovian and instrumental memories. In pavlovian-instrumental transfer (PIT) animals are first trained by pairing a (typically auditory) CS with reward. Animals are then trained on an instrumental task whereby they must respond in order to receive the same reward as the initial pavlovian training. In the test session the ability of the CS to promote responding on the lever can be used to probe the association between the CS and the reward (see Figure 1.3C). Memories underlying this form of responding have been shown to undergo reconsolidation. NMDA receptor antagonism during reactivation sessions consisting of presentation of the CS result in deficits in this behaviour (Lee and Everitt, 2008c). The ability of a CS to promote responding can also be assessed in cases where animals are required to respond on levers in order to receive delivery of drugs (or food), paired with CS delivery. In the test session reward-paired CSs are delivered in a response contingent manner; presentation of these CSs results in enhanced instrumental responding. Using this method it has been shown that administration of NMDA receptor antagonists results in decreases in seeking potentiated by cocaine-paired cues (Milton *et al.*, 2008a). Using similar methods it has been shown that reconsolidation of food-paired CSs reconsolidates in a similarly NMDA receptor-dependent manner (Flavell and Lee, 2013; Lee and Everitt, 2008b). The involvement of NMDA receptors in reconsolidation can also be demonstrated through the administration of agonists of these receptors prior to reactivation resulting in an enhancement in reconsolidation. Treatment with the partial NMDA receptor agonist d-cyloserine (DCS) prior to reactivation of a cocaine-CS memory results in a subsequent *increased* responding for this stimulus (Lee *et al.*, 2009).

Figure 1.3: Tasks used to study pavlovian appetitive memory in rodents. In each case, the left hand box shows the procedure used in training and the right used at test. Green arrows represent instrumental responding. Reward delivery is indicated by a pellet in the food magazine. **A**: Autoshaping: animals will approach a reward-paired CS. **B**: Conditioned reinforcement, acquisition of a new response: response contingent presentation of reward-paired cues is sufficient to support acquisition of an otherwise non-reinforced instrumental response. **C**: Pavlovian to instrumental transfer (PIT): delivery of a reward-paired CSs results in increased instrumental responding. Speaker symbol represents delivery of an auditory stimulus. **D**: Relapse procedure: delivery of reward-paired CSs results in enhanced responding of a previously reinforced instrumental response. Responding in this procedure may be mediated by pavlovian approach to the reward-paired CS, conditioned reinforcement and/or PIT, although not necessarily in equal measure. **E**: Venn diagram showing the suggested memory mechanisms tested with these tasks.

NMDA receptors are also required for fear memory reconsolidation, with systemic administration of NMDA receptor antagonists prior to retrieval of cued (Lee *et al.*, 2006b; Merlo *et al.*, 2014) or contextual fear (Heath *et al.*, 2015) memories resulting in decreased freezing in tests conducted the next day. Pre-reactivation administration of DCS results in increased fear expression in subsequent tests, suggestive of an enhancement in reconsolidation (Lee *et al.*, 2006b; Merlo *et al.*, 2014).

The NMDA receptor is comprised of two subtypes, which appear to have divergent roles in some memory processes. The subtypes GluN1 and GluN2 (historically referred to as NR1 and NR2). The GluN2 receptors have four subunits, GluN2A-D, with the GluN2A and GluN2B receptor subunits being most widely studied. These have been shown to have differential electrophysiological properties, with GluN2A receptors required for LTP and GluN2B receptors required for LTD both within the hippocampus (Liu *et al.*, 2004) and the inputs from the auditory thalamus to the lateral amygdala (Dalton *et al.*, 2012). These receptor subtypes have distinct roles in memory acquisition, with GluN2A, but not GluN2B receptors required for fear memory acquisition (Dalton *et al.*, 2012), although GluN2B receptors do appear to be engaged in this process for strong fear memories (Zhang *et al.*, 2008).

GluN2A and GluN2B receptors also have contrasting roles in memory reconsolidation. As described previously, administration of broad spectrum NMDA receptor antagonists prior to memory retrieval can result in deficits in memory reconsolidation. Somewhat paradoxically, however, in 2006 it was demonstrated that pre-reactivation infusion of AP-5, a non-selective NMDA receptor antagonist prevents the destabilisation of pavlovian fear memories. Through the use of a double infusion procedure, where drugs were administered directly into the BLA both before and after memory reactivation sessions, it was demonstrated that infusion of NMDA receptor antagonists prior to memory retrieval results in a resistance to the amnestic effects of post-reactivation infusion of the protein synthesis inhibitor anisomycin (Ben Mamou *et al.*, 2006). Without further resolution it was not clear how the amnestic effects of broad-spectrum NMDA receptor antagonists were mediated; administration of these drugs should prevent destabilisation, thus preventing any interference of reconsolidation (a process that cannot take place without prior memory reactivation). However, in the Ben Mamou *et al.* study the NMDA receptor antagonists used were either non-subunit specific (AP-5) or only antagonised GluN2B subunits of this receptor (ifenprodil). Further investigations in Milton *et al.* (2013) using similar methods replicated the effect of GluN2B antagonism on memory destabilisation, but it was also shown that antagonism of GluN2A receptors results in amnesia when given in conjunction with memory reactivation session, even in the absence of protein synthesis blockade. Thus, whilst NMDA receptors are required for both destabilisation

and reconsolidation of auditory fear memories, different subunits of these receptors mediate these two processes. GluN2B receptor subunits are required for memory destabilisation and GluN2A for reconsolidation. This raises the possibility that the amnestic effects of the administration of broad spectrum NMDA receptor antagonists occur as a result of their action upon GluN2A receptors subunits and the destabilisation-preventing effects of these drugs are mediated by GluN2B antagonism (Milton *et al.*, 2013). It should be acknowledged, however, that it is possible that administration of NMDA receptor antagonists prevents the pharmacological action of anisomycin, rather than exerting its effects through prevention of memory destabilisation. This possibility could be investigated with GluN2B receptor antagonism following memory reactivation (when destabilisation should have taken place). If GluN2B receptor activation is specifically required for destabilisation post-reactivation ifenprodil treatment should have no impact on the ability of subsequent protein synthesis blockade to prevent reconsolidation.

AMPA and kainate receptors

AMPA receptors are most likely required for memory retrieval, but not consolidation or reconsolidation. Binding of extracellular glutamate to AMPA and kainate receptors results in the opening of these channels, resulting in the entry of sodium and the exit of potassium. Whilst infusion of AMPA receptor antagonists within the hippocampus has no effect on acquisition, the same treatment impairs retrieval of a spatial memory (Bast *et al.*, 2005; Liang *et al.*, 1994). Similarly, infusion of these drugs appears to prevent retrieval, but not acquisition, of an association between a cocaine drug-paired stimulus, as measured by the ability of this cue to permit acquisition of a new response (Cardinal *et al.*, 2003). Finally, infusion of AMPA receptor antagonists into the BLA prevents the expression, but not reconsolidation, of fear memories (Milton *et al.*, 2013). Kainate receptors have received minimal attention in the re/consolidation literature, likely the result of a paucity of drugs targeting this system without simultaneous antagonism of AMPA receptors.

Metabotropic glutamate receptors

Very few studies have investigated the role of mGluRs in consolidation or reconsolidation. Local infusion of mGluR1, but not mGluR5 receptor antagonists into hippocampus immediately following a contextual-fear memory training session prevents subsequent expression of these memories (Maciejak *et al.*, 2003). Antagonism of mGluR1 and mGluR5 receptors has been shown to prevent consolidation and reconsolidation of inhibitory avoidance tasks in the day-old chick (Gieros *et al.*, 2012).

GABA

Alongside glutamate, which exerts its effects via increasing the likelihood of synaptic activity occurring, the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) is also important in memory consolidation and reconsolidation. Systemic administration of agonists of this system impairs acquisition of inhibitory avoidance (Jensen *et al.*, 1979) and contextual fear (Harris and Westbrook, 1998) memories, effects suggested to be distinct from the anti-anxiolytic properties of these drugs (Pain *et al.*, 2002). The deficits occurring as a result of hippocampal infusions of GABA receptor agonists are only apparent 6 or more hours following acquisition. This suggests these effects appear to be, at least in part, caused by the effects of GABA agonism prevent memory consolidation, rather than the result of global inactivation of the hippocampus resulting in an impairment in the encoding of the context during acquisition (Misane *et al.*, 2013).

Whilst there have been very few, if any, reports of the role of GABA receptors in consolidation of reward-related memories, administration of the $GABA_A$ receptor agonist midalozam prevents the reconsolidation of morphine conditioned place preference (Robinson and Franklin, 2010). This same drug also prevents reconsolidation of aversive pavlovian memories (Bustos *et al.*, 2006).

Noradrenaline

The noradrenergic system has long been implicated in the regulation of memory. Memory enhancements occurring as a result of post-trial treatment with adrenaline, corticosterone or agonists of these systems are prevented by co-administration of β-adrenergic receptor antagonists (Gold and Buskirk, 1978; Gold and Buskirk, 1975; Liang *et al.*, 1986; Roozendaal *et al.*, 2006a; Roozendaal *et al.*, 2006b; Roozendaal *et al.*, 2008; Sternberg *et al.*, 1985; Wichmann *et al.*, 2012; Zorawski and Killcross, 2003). The role of these receptors in consolidation in the absence of these memory enhancing treatments appears to be more task specific. Whilst β-adrenergic receptor antagonism prevents acquisition of passive avoidance tasks (Gallagher *et al.*, 1977), the same treatment does not affect consolidation of pavlovian associations with aversive stimuli (Bush *et al.*, 2010; Dębiec and Ledoux, 2004) they are required for reconsolidation of these associations. Systemic injections of the β-adrenergic receptor antagonist propranolol prevents the reconsolidation of both auditory CS (Dębiec and Ledoux, 2004) and contextual (Muravieva and Alberini, 2010; Taherian *et al.*, 2014) fear memory reconsolidation. Antagonism of these receptors within the BLA also results in similar deficits in auditory fear memory reconsolidation (Dębiec and Ledoux, 2004).

Both the consolidation and reconsolidation of appetitive associations relies upon β-adrenergic receptors, with administration of propranolol immediately following acquisition (Bernardi *et al.*, 2006) or reactivation (Bernardi *et al.*, 2009; Robinson and Franklin, 2007a) of a drug-conditioned place preference memory resulting in subsequent deficits in memory expression. Research has also been conducted investigating the role of reward-related memories in tasks that allow the specific contribution of pavlovian associations to performance to be assessed. Treatment with propranolol prior to memory reactivation has been shown to disrupt reconsolidation of a CS-US memory. This results in decreased conditioned reinforcing properties the reactivated CS, as manifested by an inability of reward-paired cues to permit ANR (Milton *et al.*, 2008b; Schramm *et al.*, 2016). In contrast to NMDA receptor antagonism, treatment with this drug at memory reactivation, has no effect on the subsequent expression of PIT and conditioned approach behaviour (Lee and Everitt, 2008c; Milton *et al.*, 2012). These differences have been cited as being the result of the these tasks relying on differing neural structures. Whilst the conditioned reinforcing properties of a CS when measured with ANR depend upon the BLA (Burns *et al.*, 1993), conditioned approach and PIT rely upon the central nucleus of the amygdala (CEN) (Cardinal *et al.*, 2002; Hall *et al.*, 2001b; Parkinson *et al.*, 2000) (although the reliance of the CEN in PIT is parameter specific (Corbit and Balleine, 2005)). It appears that propranolol is only able to disrupt reconsolidation of memories dependent on the BLA, but not the CEN, possibly due to innervation of the BLA (Woolf and Butcher, 1982), but not CEN (Aston-Jones *et al.*, 1986). These regions have previously been shown to have distinct roles in the representation of the sensory-specific and general motivational properties of CSs (Balleine and Killcross, 2006). Given that the BLA appears to be the crucial in the former (Corbit and Balleine, 2005), propranolol may only be able to disrupt associations of this nature.

Dopamine

Not all events will be remembered for a lifetime and those that are associated with motivationally significant events are more likely to lead to the formation of an enduring memory. The coding of the significance of these events may depend upon activation of the dopaminergic system. Single-cell recordings from the ventral tegmental area (VTA), a region responsible for dopaminergic innervation throughout the mesolimbic system, in behaving macaques suggest that dopaminergic cells may act to signal salient events, firing in response to presentation of both appetitive and aversive unconditioned stimuli, particularly when these are unexpected (discussed further below, Mirenowicz and Schultz, 1996). In support of this notion, systemic or local infusion of dopamine receptor antagonists within the amygdala or hippocampus (Guarraci *et al.*, 1999; Heath *et al.*, 2015) prior to fear

conditioning sessions results in deficits in fear memory acquisition. Infusion of these drugs prior to learning of an appetitive pavlovian memory into both of these structures (Andrzejewski and Ryals, 2016) or the nucleus accumbens (Di Ciano *et al.*, 2001) also results in deficits in the reward-paired CS to support autoshaping and ANR, respectively. The deficits arising in these tasks appear to be the result of disrupting acquisition (in accord with the role of dopamine to attribute salience to events during learning), rather than consolidation, since administration of dopamine receptor antagonists *following* fear training has no effect on subsequent memory expression (Heath *et al.*, 2015; Inoue *et al.*, 2000).

Like noradrenaline, dopamine does, however, appear to have a role in the mediation of the effect of corticosterone to enhance consolidation. Liao *et al.* (2013) demonstrate that dopamine D2 receptor antagonism within the hippocampus following acquisition of a contextual fear memory is without effect on subsequent fear memory expression. However, when corticosterone was administered prior to training, the resulting enhancement in fear was prevented with post-training infusion of dopamine receptor antagonists (Liao *et al.*, 2013).

Only a small number of studies have explored the role of dopamine in reconsolidation, with contradicting results. Heath *et al.* (2015) report that dopamine receptor antagonism either prior to, or immediately following, memory reactivation has no effects on reconsolidation. Whilst systemic post-retrieval dopamine receptor antagonism prevents the reconsolidation of an appetitive pavlovian memory (Yan *et al.*, 2014), pre-reactivation dopamine receptor antagonism within the BLA has no effect on subsequent expression (Merlo *et al.*, 2015). Whilst caution must be applied in comparison of these two studies conducted using different reinforcers, species, routes of drug administration and memory testing conditions, the discrepancy in the amnestic effects of these two studies may lie in differences in the timing of drug administration and the hypothesised role of dopamine memory destabilisation.

Administration of dopamine receptor antagonists following memory reactivation resulted in impairments in memory reconsolidation in Yan *et al.* (2014), whilst pre-retrieval infusions of these drugs in Merlo *et al.* (2015) had no effect on the reconsolidation of the memory. Although pre-reactivation intra-BLA infusion of dopamine receptor antagonists did not prevent reconsolidation in Merlo *et al.* (2015) they were not without effect. Administration of these drugs resulted in an insensitivity to the otherwise amnestic effects of post-reactivation infusions of anisomycin, suggesting a role for the dopaminergic system in the destabilisation of these memories. Furthermore,

dopaminergic receptor antagonism in the VTA prevents the ability of a memory reactivation session to result in destabilisation of an appetitive memory (Reichelt *et al.*, 2013). The effect of pre-reactivation dopamine receptor antagonism to prevent memory destabilisation in the Merlo *et al.* paper may have prevented the amnestic effect of this treatment – the memory was not able to become destabilised this may have masked any effects on reconsolidation this drug may otherwise had. The fact that dopaminergic receptor antagonism is without effect on fear memory reconsolidation may suggest a differential involvement of this system in aversive memory reconsolidation, although further research is required on the topic.

Molecular mechanisms of reconsolidation and consolidation

The molecular basis of consolidation, reconsolidation and their constitutive processes has been extensively investigated. Given that these are not a focus of this thesis, only a brief outline of the key mechanisms implicated in these processes is outlined.

Protein synthesis

First and foremost, both memory consolidation and reconsolidation require protein synthesis. Frequently used as a hallmark test as to whether these processes are taking place, the dependence of memory consolidation on the synthesis of new proteins has been demonstrated in a wide range of tasks. Both passive (Quevedo *et al.*, 1999) and active (Agranoff *et al.*, 1966; Flood *et al.*, 1975) avoidance and discrete and contextual (Schafe and LeDoux, 2000; Schafe *et al.*, 1999) fear memories can be disrupted by post-training infusion of protein synthesis inhibitors. Reward-associated memories appear to be similarly reliant on this process, since disruption of protein synthesis following acquisition of conditioned place preference (Robinson and Franklin, 2007b), pavlovian conditioned approach (Blaiss and Janak, 2007) and instrumental learning (Hernandez and Kelley, 2004; Hernandez *et al.*, 2002, although see Jonkman and Everitt, 2009; Jonkman and Everitt, 2011) tasks disrupts subsequent performance.

The requirement for the synthesis of new proteins appears to be similarly important for memory reconsolidation as consolidation. Reconsolidation of aversive memories can be inhibited with post-reactivation administration of anisomycin in both fear conditioning (Frankland *et al.*, 2006; Nader *et al.*, 2000) and avoidance tasks (Fukushima *et al.*, 2014). Similar results have also been reported in

conditioned place preference (Robinson and Franklin, 2007b) and appetitive pavlovian memories (Merlo *et al.*, 2015).

Whilst protein synthesis inhibition has been used extensively as a tool to demonstrate the presence of both consolidation and reconsolidation processes, this approach is not without fault. Anisomycin has several non-specific effects, most notably increases in catecholamine levels at the site of infusion (e.g. Qi and Gold, 2009). Importantly, it appears that these increases in neurotransmitters, particularly noradrenaline, are responsible for some of the amnestic effects of anisomycin, rather than the effect of this drug to prevent protein synthesis; co-administration of noradrenaline receptor antagonists attenuates the effects of anisomycin on memory consolidation (Canal *et al.*, 2007; Qi and Gold, 2009). Central infusion of this drug in combination with food-delivery can also result in an aversion to the delivered reinforcer, interfering with task-performance (Jonkman and Everitt, 2009; Jonkman and Everitt, 2011) potentially leading to erroneous assumptions as to the role of protein-synthesis in the infused region to memory processes.

Extracellular signal-regulated kinase

Partly with the non-specific effects of protein synthesis blockade in mind, studies have also sought to investigate the myriad of specific molecular processes required for consolidation. The extracellular signal-regulated kinase (ERK)-mitogen-activated protein kinase (MAPK) pathway has received extensive attention in the consolidation and reconsolidation literature. ERK inhibition in the BLA results in impairments in fear memory consolidation (Schafe *et al.*, 2000). ERK activation within the nucleus accumbens core is required for cocaine conditioned-place preference (Miller and Marshall, 2005), although in this study all infusions were administered prior to conditioning sessions, raising the possibility the results were caused by a prevention of the reinforcing effects of cocaine. However, in a similar study conducted on amphetamine conditioned-place preference it was revealed that post-training infusions of ERK inhibitors similarly result in deficits in the acquisition of conditioned-place preference (Gerdjikov *et al.*, 2004). ERK has a demonstrable role in aversive and appetitive reconsolidation. Inhibition of this kinase within the BLA prevents auditory fear (Duvarci *et al.*, 2005), inhibitory avoidance (Krawczyk *et al.*, 2016) and conditioned-place preference (Valjent *et al.*, 2006) memory reconsolidation.

Immediate early gene zif-268

The immediate early gene (IEG) *zif-268* (also known as *EGR1*, Krox4) also has a critical role in reconsolidation. Expression of this gene is increased in the BLA following reactivation of discrete fear memories (Hall *et al.*, 2001a; Tedesco *et al.*, 2014b), contextual fear memories in the hippocampus (Hall *et al.*, 2001a; Lee *et al.*, 2004) and in the BLA following exposure to a cocaine paired stimulus (Thomas *et al.*, 2003), an effect mediated by upstream NMDA receptor activation (Milton *et al.*, 2008a). These increases appear to be integral to the maintenance of the reactivated memory since intra-hippocampal/BLA infusion of *zif-268* oligodeoxynucleotides (ODNs) prior to reactivation of each of these memories, respectively, results in deficits in subsequent expression (Lee *et al.*, 2004; Lee *et al.*, 2005b).

Interestingly, there is less evidence for a requirement of *zif-268* in memory consolidation. Knockdown of this gene in the dorsal hippocampus prior to a contextual fear training session consisting of two footshocks had no effect on subsequent acquisition. However, when the training was divided into two sessions, a day apart, such that the memory of the first day of training was fully consolidated, infusion of *zif-268* ODNs resulted in a failure to increase the strength of the memory in this second training session via reconsolidation mechanisms (Lee *et al.*, 2004). Thus, whilst expression of *zif-268* in the hippocampus is not required for the consolidation of contextual fear memories, the updating of the same memory via reconsolidation mechanisms depends, at least in part, to rely on distinct cellular processes. The opposite is true of brain-derived neurotrophic factor (BDNF), with this protein being required for the consolidation, but not reconsolidation of these memories (Lee *et al.*, 2004). The formation and updating of existing memories is therefore carried out via distinct cellular mechanisms, suggesting that reconsolidation is not simply re-consolidation.

Mammalian target for rapamycin (mTOR) pathway

The mammalian target of rapamycin (mTOR) pathway is a protein kinase downstream of several neurotransmitter systems, including NMDA receptors, that is responsible for controlling the assembly of mTOR complexes. These regulate protein translation and activity of phosphates critical for synaptic plasticity (Hoeffer and Klann, 2010). mTOR inhibition both systemically and within the BLA and dorsal hippocampus results in impairments in the consolidation of contextual fear memories (Gafford *et al.*, 2011; Parsons *et al.*, 2006). Systemic and intra-BLA administration of rapamycin

within the BLA also impairs consolidation of discrete cued fear memories (Gafford *et al.*, 2011). Reconsolidation of these memories is similarly dependent on mTOR, with systemic rapamycin preventing reconsolidation of cued and contextual fear (Blundell *et al.*, 2008; Hoffman *et al.*, 2015). Local inhibition of the mTOR pathway within the BLA (Parsons *et al.*, 2006) and dorsal hippocampus (Gafford *et al.*, 2011) also prevents the reconsolidation of cued and contextual fear memories, respectively. Whilst the requirement of rapamycin in the reconsolidation of reward-related memories has only received minimal attention in the literature, evidence suggests that systemic rapamycin following reactivation of a morphine, cocaine or alcohol conditioned place preference memory prevents its subsequent expression (Lin *et al.*, 2014). Disruption of mTOR signalling within the CEN in conjunction with a memory reactivation session also results in decreased alcohol seeking (Barak *et al.*, 2013), although the specific memory targeted by this procedure is unclear (discussed further below).

Protein degradation

Investigations have also focussed their efforts on the molecular mechanisms underlying the destabilisation of memories, which likely depends on the ubiquitin-proteasome system, required for proteasome-dependent proteolysis (Jarome and Helmstetter, 2013). The destabilisation of contextual fear memories results in increased polyubiquitination of scaffolding proteins Shank and GKAP in the dorsal hippocampus and infusion of the proteasome inhibitor clasto-Lactacystin-β-lactone (β-lac) prevents the otherwise amnestic effect of post-retrieval anisomycin (Lee *et al.*, 2008).

Similar effects have been reported within the BLA; reactivation of a pavlovian fear memory results in an increase in markers of protein polyubiquitination in this region and infusion of β-lac in this region protects these memories from the reactivation dependent amnestic effects of anisomycin infusion directly into the BLA (Jarome *et al.*, 2011). The ubiquitin-proteasome system may interact with previously discussed mechanisms of destabilisation, perhaps most notably GluN2B activation (Ben Mamou *et al.*, 2006; Milton *et al.*, 2013).

Psychology of memory reconsolidation

Whilst preventing the pharmacological and molecular processes required for reconsolidation to take place results in impairments in this process, these manipulations are without effect if the memory has not been reactivated: the requirements for memory reactivation are not only biological,

but also psychological. Not all retrieval trials will result in reconsolidation and the psychological determinants of this process taking place are outlined below.

Function

The requirements for memory consolidation to take place are relatively simple, the key determinant in whether this process occurs is memory formation. Detecting whether this process has occurred is, therefore, simple; any session that results in a change in behaviour as a result of new learning has, most likely, triggered the process of consolidation. In contrast, reconsolidation is typically a silent process; sessions that trigger reconsolidation may have no impact on the subsequent expression of the memory. Furthermore, memory retrieval is neither necessary nor sufficient to result in reconsolidation of a memory (Alfei *et al.*, 2015; Díaz-Mataix *et al.*, 2013; Milton *et al.*, 2013). Whether reconsolidation takes place is therefore frequently determined by the ability of a retrieval session to result in susceptibility of the memory trace to amnestic agents, providing evidence that the association has been destabilised.

Many factors relating to both the training that led to the formation of the memory trace, the memory retrieval session and interactions between these two affect the ability of a retrieval session to result in memory destabilisation. In order to understand the conditions that might lead to memory reconsolidation occurring, one must consider why this process exists in the first place. It could be argued that repeated exposure of a memory to interference may put organisms at an evolutionary disadvantage. Despite this, the memory process appears to be remarkably conserved, having been demonstrated in numerous species (Nader, 2015; Tronson and Taylor, 2007). Memory reconsolidation may serve to ensure the maintenance of significant memories (Tronson and Taylor, 2007). Memory reactivation can result in memory strengthening (Fukushima *et al.*, 2014; Rohrbaugh and Riccio, 1970; Tedesco *et al.*, 2014b) and a resistance to forgetting (Inda *et al.*, 2011). Memory reconsolidation may also exist to allow previously formed memories to be updated with new information (Lee *et al.*, 2009; Nader and Einarsson, 2010). This hypothesis has received much empirical support, evidence for which is outlined below.

Prediction error

In order for a memory to be updated, novel information must be present at retrieval. At its simplest, this takes the form of prediction error (PE), where there is disparity between the outcome expected

and that which is obtained. This has been formally conceptualised by Rescorla and Wagner (1972, see Equation 1.1) where ΔV, the change in conditioning strength is determined by α and β, the rate parameter of the CS and US, respectively, the maximum conditioning that can be obtained by the US (λ) and the current sum of all associative strengths (ΣV).

$$\Delta V = \alpha\beta(\lambda - \Sigma V) \tag{1.1}$$

On the first trial, where, for example, a CS is paired with a US, the difference between λ and ΣV is maximal, since this latter value is zero (no learning has taken place). However, on the second trial the ΣV has been updated to include learning which occurred on the first trial, and the difference between λ and ΣV is reduced. As multiple trials take place, the ΣV value is almost equal to λ, such that no further learning will take place.

Retrieval sessions that result in a large discrepancy between the expected (ΣV) and obtained outcome (λ) will result in PE (large ΔV). This has been shown to affect the likelihood of a retrieval session to result in memory destabilisation. In the crab *Chasmagnathus* reconsolidation is only triggered when the expected US is omitted in a retrieval trial (Pedreira *et al.*, 2004). In human subjects trained to fear a visual stimulus, where each image presentation is paired with shock, a single presentation of the CS in the absence of the US is sufficient to result in reconsolidation that is sensitive to antagonism of β-adrenergic receptors (Sevenster *et al.*, 2013). In contrast, CS presentations alongside the US, as in training, have no effect on the lability of the memory. As was confirmed with online ratings of US expectancy, subjects were expecting to receive a shock when the visual stimulus was presented, and thus there was only a PE for subjects when the shock was not presented. The reliance of this effect on PE, rather than just shock presentation, was demonstrated in a third group, who only received shock pairings on every second CS presentation, and were explicitly informed this was the case. When these subjects were presented with a single CS alongside the shock, reconsolidation did take place, the expectation that only the second CS presentation would be reinforced was violated (Sevenster *et al.*, 2013). In rats trained to expect a shock after a given duration following CS presentation, only trials that violate this expectancy result in reconsolidation (Díaz-Mataix *et al.*, 2013). Finally, in contextual fear, where animals can learn to expect a shock after a certain duration after being placed in a context, only memory reactivations that are sufficient in duration to result in the experience of a PE trigger reconsolidation (Alfei *et al.*, 2015). Thus, US presentation is not the sole determinant as to whether this process takes place; instead it is the degree to which this was expected.

One likely neural candidate for these PE signals is the VTA, a region responsible for dopaminergic innervation throughout the mesolimbic system. The firing rate of neurons within this region is increased in response to presentation of reward when it is unexpected, but as reward delivery becomes more predictable these neurons decrease their activity in response to reward, instead showing increases in response to the presentation of the stimuli that predicts reward. Importantly, cells in this region show decreased firing in response to the omission of an expected reward (Schultz *et al.*, 1997). Whilst initially it was suggested that the increases in dopaminergic firing to CSs were selective to appetitive stimuli, recent evidence has demonstrated similar increases in dopaminergic firing in response to stimuli predictive of aversive events (Matsumoto and Hikosaka, 2009).

These findings have lent support to the notion that the increases and decreases in dopamine release may be used to inform the unexpected presentation and absence of reward, respectively. If PE is indeed required for a retrieval trial to result in reconsolidation, and these signals do indeed reflect this, disruption of dopamine receptor activation should prevent reactivation sessions resulting in labilisation of the memory. Whilst presentation of a CS paired with reward can result in reconsolidation of an association between these two stimuli, disruption of dopaminergic signalling within the VTA prevents this destabilisation process. Administration of a dopamine receptor antagonist within this region results in a loss of the amnestic effect of NMDA receptor antagonism (Reichelt *et al.*, 2013). The effects of the reward sensitive responses of the VTA are also likely expressed in many of the structures that this region projects to, including the amygdala (Swanson, 1982). Dopaminergic signalling within this region also appears to be important in determining whether retrieval results in memory destabilisation. Administration of either dopamine D1 or D2 receptor antagonists within the BLA prior to memory retrieval preventes the effect of post-trial administration of anisomycin to prevent the reconsolidation of a CS-reward association (Merlo *et al.*, 2015). Whilst both of these studies support the view that activation of dopamine receptors is required for destabilisation, whether this is the result of disrupted PE signals is unclear; neither of these studies investigated whether the ability of the memory retrieval session to result in destabilisation could be prevented through delivery the expected reinforcer and whether such a manipulation led to a loss of the sensitivity to dopamine receptor antagonism. More conclusive evidence for the role of dopamine in signalling PE that leads to destabilisation might involve demonstration that administration of dopamine receptor agonists enable a retrieval session with minimal PE that otherwise does not lead to reconsolidation to result in destabilisation. It would be expected that whilst dopamine receptor agonism would have such an effect, other treatments shown to potentiate destabilisation,

but not PE, such as cannabinoid CB1 receptor agonism (Lee and Flavell, 2014) or perhaps GluN2B receptor stimulation (Milton *et al.*, 2013) would not.

There is a wealth of evidence suggesting that PE is a critical determinant as to whether a retrieval trial results in destabilisation, as measured by an ability of amnestic agents to prevent the subsequent reconsolidation of these memories. Retrieval trials that do not trigger PE do not result in these processes, and this is true for both appetitive and aversive memories. Blockade of neural signatures hypothesised to mediate these signals also has similar effects for appetitive memories. However, if PE was the only critical determinant in whether a memory retrieval session results in its reactivation, strong fear memories should rapidly destabilise with a single CS presentation. Multiple training sessions should have allowed the difference between the maximum possible association from the US (λ) and the associative strength with the CS (ΣV) to be minimal, thus when λ changes for a given trial as a result of the omission of the US the difference between these two values is high. However, this has been shown not to be the case. For example, Suzuki *et al.* (2004) report that memories resulting from a single contextual fear training session will destabilise, as indicated by a susceptibility to systemic injections of anisomycin, after a 3-minute retrieval trial without shock. The same is not true for memories having undergone multiple pairings, with anisomycin treatment following a retrieval session of the same duration now being without effect. However, a 10-minute reactivation was effective at inducing destabilisation of these strong memories. There appears to be an interaction between memory strength and the PE required for destabilisation to occur; as memory strength increases the PE required to result in destabilisation increases twofold – longer reactivation sessions are required, which should result in a greater number of opportunities to experience the surprising absence of a shock, but also the magnitude of this surprise should be greater, given the high ΣV value obtained in training. The greater PE required to destabilise stronger memories likely relates to the function of reconsolidation to update previously formed memories. If a stimulus has been paired with an aversive event many times, a single occurrence whereby this event does not occur should not be sufficient to override the previous learning. A retrieval trial that suggests an aversive event follows CS presentation 99%, rather than 100% is unlikely to warrant the triggering of memory updating mechanisms. In contrast, a retrieval trial that suggests that the CS is reinforced 50%, rather than 100% of the time is more likely to result in memory destabilisation.

Whilst there appears to be a minimum level of PE required to trigger reconsolidation, memory reactivations that consist of a large number of opportunities to experience a violation of expectation

will also not result in memory destabilisation. In auditory fear conditioning, for example, presentation of a single CS without the expected shock can result in a reactivation of the memory. However, if the CS is presented multiple times without reinforcement a different process, extinction, will occur (Lee *et al.*, 2006b; Merlo *et al.*, 2014). Whilst initial pavlovian learning results in an association between a stimulus and its outcome, extinction learning results in the formation of a new inhibitory memory opposing the initially formed association. Trials that result in reconsolidation and extinction therefore have separate behavioural outcomes; the former typically leaving the initial memory unaltered (without pharmacological intervention) and the latter resulting in a decrease in subsequent expression of the memory.

Whether a retrieval trial will result in extinction or reconsolidation is typically determined by their length, or the number of opportunities for the violation of learned expectations. Shorter retrieval trials, consisting of fewer CS presentations (in discrete CS fear conditioning), will result in reconsolidation, whilst longer retrieval trials lead to extinction. Until recently, however, the processes are engaged in sessions in retrieval trials in between these two extremes have been unclear. This phenomenon has been investigated by Merlo *et al.* (2014) in discrete fear memories. Here it was demonstrated that a single CS presentation results in reconsolidation that is susceptible to NMDA receptor antagonism – this treatment results in a decrease in responding in a test session the following day. Retrieval trials consisting of 10 CS presentations resulted in extinction, as evidenced by a decrease in responding both within the retrieval session and the test session the following day (in the absence of any pharmacological intervention). Administration of an NMDA receptor antagonist prior to this type of retrieval session resulted in impairments in extinction learning, as indicated by an increase in freezing the next day in comparison to vehicle treated controls (Merlo *et al.*, 2014). NMDA receptor antagonism prior to presentation of an intermediate number of CSs (4), however had no effect on memory expression, suggesting that neither reconsolidation nor extinction was being triggered by retrieval sessions of this nature and the memory was in 'limbo' (Merlo *et al.*, 2014). Recent investigations have also analysed levels of phospho-ERK1/2, following retrieval, demonstrating that sessions consisting of 1 or 10 CS presentations result in an increase in this kinase. However, sessions consisting of an intermediate number of CSs have no impact on pERK1/2 levels, supporting the notion that neither reconsolidation nor extinction was being engaged by these retrieval trials (Merlo *et al.*, in preparation).

The requirements for PE within a reactivation session to result in reconsolidation are, therefore, highly specific. If the retrieval session is too similar to acquisition and the memory need not be updated, it will not be destabilised. As PE increases reconsolidation processes will indeed be engaged.

If there is too much, then the window for reconsolidation will close, with the initial engagement of a limbo phase, followed by extinction. Trials of an intermediate duration have no impact on memory expression, in the presence or absence of pharmacological agents, whilst decreases in responding can be observed either through extinction or disruptions of reconsolidation. Whilst these two latter memory manipulations both result in a decrease in responding, the resulting changes in behavioural expression likely occur through distinct mechanisms.

There are several lines of evidence that suggesting extinction is the result of new learning, rather than erasure of the original trace, typified by the ability of the original memory to return as a result of several manipulations. For example, following a period of extinction of a CS-US memory in a context distinct from that used in training, returning animals to the training context, or even a novel context, can result in *renewal* of responding (see Figure 1.4A). Presentation of the US results in *reinstatement* of responding after extinction (see Figure 1.4B), the passage of time between extinction and test results in *spontaneous recovery* (see Figure 1.4C). Upon a reminder trial, where the once extinguished association is once again reinforced, animals can also show accelerated *reacquisition* of this association, in comparison to naïve controls (see Figure 1.4D; Bouton, 2002).

The ability of once extinguished memories to regain control over behaviour after these manipulations represents an important difference between a decrease in behavioural expression resulting from disruptions from reconsolidation and extinction. Whilst the latter is sensitive to mechanisms of the return of memory expression depicted in Figure 1.4, the same is not true of associations where memory reconsolidation has been disrupted; decreases in responding occurring as a result of disrupted reconsolidation are not context-dependent, not sensitive to reinstatement and do not show spontaneous recovery (Duvarci and Nader, 2004; Kindt *et al.*, 2009).

Retrieval-extinction procedures

Manipulating the manner in which an extinction session occurs can apparently result in a decrease in responding that is insensitive to sources of relapse described above. Monfils *et al.* (2009) report that the addition of a reactivation of a discrete CS-fear memory prior to its extinction results in a decreased freezing that is context-independent, impervious to reminder shocks, the passage of time and results in retarded re-acquisition in comparison to animals that have received no retrieval trial prior to the extinction sessions. Further investigations into this effect have shown similar results can be obtained with contextual fear (Flavell *et al.*, 2011; Rao-Ruiz *et al.*, 2011), morphine and cocaine induced conditioned place preference (Sartor and Aston-Jones, 2014; Xue *et al.*, 2012) and

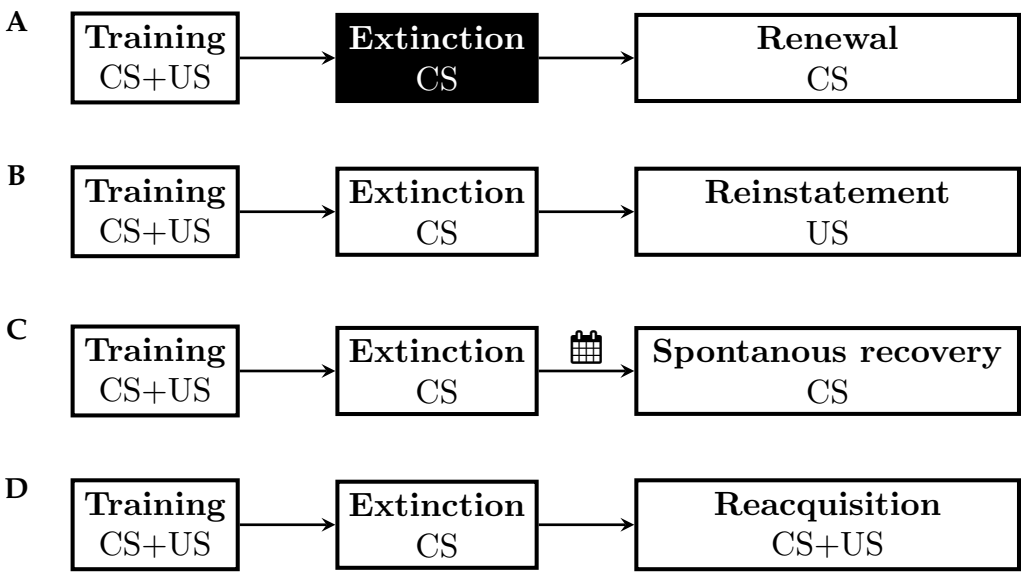


Figure 1.4: Four possible methods in which extinguished associations can once again take control of behaviour. Although pavlovian associations are depicted here, the same processes occur for instrumental associations, where, in this diagram, the CS would be replaced with an opportunity to make an instrumental response. **A**: Returning animals to a context they have been trained in after a period of extinction in a novel context can result in renewal. Note that the context is not explicitly paired with an outcome – it is a discrete CS (or instrumental response) that is associated with a reinforcer. Renewal can also occur upon shifting to a novel context for the test session. **B**: Presentation of the US alone can result in reinstatement of responding. **C**: Passage of time following extinction results in spontaneous recovery. **D**: Reminder trials of the once extinguished association can result in rapid reacquisition of responding. Adapted from Bouton (2002).

CS-heroin memories formed during self-administration in rats, with retrieval trials prior to extinction of these CS memories resulting in decreased propensity to show relapse-like behaviour in later tests (Xue *et al.*, 2012). Whilst the mechanism for these effects is the topic of much ongoing debate (McNally and Hutton-Bedbrook, 2013), the preferred explanation from many of those advocating this procedure is that the retrieval trial results in the destabilisation of the memory, priming it for the incorporation of new information. In the following extinction trials the original memory is updated with the information that the CS no longer predicts the US, rather than a new inhibitory memory being formed with this information (Monfils *et al.*, 2009). Alongside the behavioural evidence suggesting that extinction trials of this nature are different from 'normal' extinction, there is also accumulating evidence that distinct brain mechanisms are recruited by retrieval-extinction procedures. For example, retrieval and retrieval-extinction, but not extinction alone procedures results in up-regulation of *zif-268* in the medial prefrontal cortex and amygdala (Tedesco *et al.*, 2014a).

Delivery of the US, like CS presentation, is also apparently sufficient to result in memory reactivation in retrieval-extinction procedures. Presentation of a shock prior to extinction of a fear memory results in similarly enhanced extinction that is resistant to many of the routes to relapse depicted in Figure 1.4 in both humans and rats (Liu *et al.*, 2014). In a similar fashion, administration of previously self-administered drugs prior to extinction of the operant response paired with these substances has been shown to prevent the renewal and reinstatement of responding (Luo *et al.*, 2015). Perhaps most remarkably in each of these studies, this US-extinction procedure not only prevented the recovery of memory expression of the extinguished CS or response, but also a second association formed with the same US but a different CS or response (Liu *et al.*, 2014; Luo *et al.*, 2015). In these US-extinction studies it is not entirely clear which memories are being targeted by the manipulations. Whilst the extinction sessions typically only involve execution of the instrumental response and not CS delivery, the test sessions involved response contingent presentation of the cocaine paired stimuli. Nonetheless these data suggest that presentation of the US results in the reactivation of an associated response or CS, and the unexpected lack of the execution of this response or presentation of the CS could result in sufficient PE to result in destabilisation of this trace (similar treatments have been shown to result in the labilisation of memories leading to their vulnerability to amnestic agents (Milekic *et al.*, 2006; Valjent *et al.*, 2006)). However, the effects on the second, non-extinguished association are more difficult to explain. It appears that the US induced retrieval (of both traces) primes these associations for modification and given the shared outcome of both these associations, any subsequent new information is equally incorporated into the two associations.

Reconsolidation based treatments for psychiatric disorders

The apparent permanence of the reductions in memory expression occurring as a result of pharmacological blockade of reconsolidation or retrieval-extinction procedures has led some commentators to (controversially) suggest these techniques result in erasure of the original trace (Clem and Huganir, 2010; Kindt *et al.*, 2009; Maren, 2011), rather than the formation of novel inhibitory memories, as is hypothesised to take place in extinction. Whilst much of the research discussed previously has provided a fascinating insight into the mechanisms underlying memory acquisition and updating mechanisms, the suggested ability to erase memories could have major implications for the treatment of psychiatric disorders.

Several psychological disorders are characterised by maladaptive, overly intrusive memories that exert a powerful and debilitating influence on an afflicted individual's life. The nature of several such disorders will now be outlined, and the potential for the use of reconsolidation based treatments as a form of therapeutic intervention for these conditions discussed.

Post-traumatic stress disorder

Post-traumatic stress disorder (PTSD) has a lifetime prevalence of approximately 7% (Kessler *et al.*, 2005), with ~15% of individuals that suffer a traumatic event going on to suffer symptoms of PTSD, although this depends on the type of trauma experienced (Helzer *et al.*, 1987; Vries and Olff, 2009). Once classified as an anxiety disorder, it is now described as a trauma and stressor-related disorder in the diagnostic and statistical manual of mental disorders (DSM)-5 (APA, 2013). Individuals with PTSD, alongside having experienced (or witnessed) a highly traumatic event, also suffer frequent, involuntary, intrusive memories of the event and suffer intense psychological distress upon perceiving cues related to such an event amongst many other symptoms (APA, 2013). The ability of these stimuli to invoke such distress presents one of the major issues for those suffering with PTSD. With this in mind, psychological therapies have attempted to reduce the impact of these cues to result in the unwelcome retrieval of these traumatic memories.

One possible method in which the ability of these cues to trigger such anxiety responses could be reduced is the pharmacological modulation of memory consolidation. Pilot studies administering the β-adrenergic receptor antagonist propranolol within 6 hours of experiencing a trauma have shown some promise in this regard (Pitman *et al.*, 2002). However, disruption of consolidation suffers from several potential drawbacks that may impede this from becoming a widespread treatment for PTSD. Any drugs would need to be given within 6h of the event, which may be impractical. In Pitman *et al.* participants were taken from an emergency room, however not all traumatic events require visits to a hospital and PTSD can also develop from not only experiencing, but also witnessing a traumatic event (APA, 2013). Access to individuals that require such a treatment may, therefore, be difficult. Furthermore, given that the majority of traumatic events will not result in the development of PTSD (VanElzakker *et al.*, 2014), it may be unclear when these treatments would be necessary. Finally, some traumas, such as those related to childhood abuse may have been formed a series of events, thus making pharmacological inhibition of consolidation difficult.

Another possible avenue for the reduction of the effects of these stimuli is cue-exposure therapy. Here, cues that elicit retrieval of a traumatic event are presented multiple times in a safe environment, degrading the association between these cues and trauma. Tapping into the well-studied process of extinction, these treatments have yielded some success (Marks *et al.*, 1998; Powers *et al.*, 2010). Furthermore, administration of drugs previously shown to enhance synaptic plasticity mechanisms, such as the NMDA receptor partial agonist DCS as an adjunct to these treatments has been shown to enhance their efficacy (Davis *et al.*, 2006; Difede *et al.*, 2014). However, even when enhanced these treatments present several drawbacks. As discussed above, extinction is hypothesised to result in the acquisition of a new inhibitory memory, rather than erasure of the original trace. This means that extinction based treatments may only be able to result in a temporary decrease in memory expression.

Given the difficulties in disruption of consolidation and cue-exposure therapy as a treatment, research has focussed on the potential use of reconsolidation based treatments to reduce the impact of maladaptive memories associated with PTSD. Whilst many of the manipulations discussed in previous sections are wholly inappropriate for human use, most notably protein synthesis inhibition and intra-cranial infusion of substances directly into the amygdala, some of the drugs shown to prevent memory reconsolidation in preclinical models are also approved for human use. One of the most widely explored possibilities in this regard is antagonism of β-adrenergic receptors. As mentioned previously, propranolol has shown to be effective at preventing memory reconsolidation in preclinical research (e.g. Dębiec and Ledoux, 2004). Administration of this drug prior to retrieval of a fear memory formed the previous day also results in markedly reduced physiological responses to stimuli associated with an experimenter administered shock in humans (Kindt *et al.*, 2009). Similar results have also been obtained following the retrieval of memories associated with PTSD (Brunet *et al.*, 2008).

In this study, participants were played an audio 'script' describing an event associated with trauma. Subjects administered propranolol immediately following this retrieval episode showed a significant reduction in physiological responses during mental imagery of traumatic events a week later, suggesting a disruption of reconsolidation. However, this finding was somewhat compromised by a lack of a group receiving propranolol in the absence of a memory reactivation session, raising the possibility the effects reported may have been the result of drug administration alone (Brunet *et al.*, 2008). Furthermore, subsequent attempts to replicate this effect from the same group have been unsuccessful (Wood *et al.*, 2015).

Preclinical studies have shown that the mTOR inhibitor rapamycin (Sirolimus) can prevent memory reconsolidation (Blundell *et al.*, 2008; Gafford *et al.*, 2011; Huynh *et al.*, 2014). However, administration of this drug prior to a script-based retrieval session had no effect on a series of clinical and physiological measures recorded at several time points after memory reactivation (Surís *et al.*, 2013). It is unclear, however, whether the lack of an amnestic effect was the result of the pharmacological intervention being unable to prevent reconsolidation. Although well characterised in preclinical models, there have been few attempts to use this drug to prevent memory processes in humans, thus raising the possibility the lack of an impairment was the result of inadequate dose, route or timing. Alternatively, it is possible that in these individuals the retrieval session was insufficient to result in memory destabilisation.

Given the difficulties of reactivation sessions not triggering reconsolidation, and perhaps more problematically, not knowing whether this is the case, recent investigations have attempted to use highly personalised reactivation sessions to increase the likelihood of a retrieval session resulting in reconsolidation. Kindt and Emmerik (2016) used interviews with PTSD sufferers to identify the most painful memories ('hot-spots') in these patients. Once identified these hot-spots were re-lived in the subsequent reactivation sessions. Once the individual reached maximal distress as a result of this, the reactivation sessions ceased, in order to prevent memories entering extinction or limbo phases and the patient administered propranolol. Whilst in an early stage of investigation, it appears these interventions can lead to a decrease in PTSD symptoms in some patients (Kindt and Emmerik, 2016).

The evidence for the treatment of PTSD remains in the early stages; studies conducted to date have typically been pilot investigations (Brunet *et al.*, 2008), and in some cases these have been unsuccessful (Surís *et al.*, 2013; Wood *et al.*, 2015). Nonetheless, complementing preclinical data, it is clear that memories underlying PTSD can become destabilised under the correct reactivation conditions and susceptible to amnestic agents.

Drug addiction

Drug addiction is a chronically relapsing disorder characterised by a cluster of cognitive, behavioural and physiological symptoms that result in continued drug abuse (APA, 2013). Whilst the criteria for diagnosis of these disorders has recently been revised, studies using the previous classification (APA, 2005) suggest 2.6% of individuals will be addicted to a substance in their lifetime (Compton *et al.*, 2007). Addicted individuals show, amongst other criteria, a markedly increased

dose of the drug required to achieve the desired effect, symptoms of withdrawal (which vary between different drugs), which are frequently relieved with resumption of drug taking, a persistent desire to cut down substance use, continued use despite adverse circumstances and the dangers it poses, and an intense urge or desire to take the drug (APA, 2013).

The progressively increased ability of drugs of abuse and their associated cues to exert control over an individual's behaviour is hypothesised to result in the recruitment of distinct neural and psychological processes. Almost all drugs of abuse share the ability to directly activate reward-related pathways (e.g. Di Chiara and Imperato, 1988). The repeated stimulation of these systems during drug abuse is suggested to play an integral role in the perpetuation of substance use disorders; opponent-process models of addiction posit that repeated stimulation of reward systems leads to their desensitisation and a shift in the 'hedonic set-point' (Ahmed and Koob, 1998). Not only does this result in escalating drug use to achieve a similar high, but increasing dysphoria when the drug is absent, which in turn can motivate continued drug seeking (Koob and Moal, 1997).

Drug-administration does not occur in sensory isolation and people, places and paraphernalia paired with drug-seeking likely become associated with the rewarding effects of the drug in a pavlovian manner; craving can be heightened by presentation of cues associated with drug taking (APA, 2013). Subsequently, exposure to these reward paired cues is a frequent cause of relapse in abstinent individuals (reviewed in Fuchs *et al.*, 2008; Taylor *et al.*, 2009, see Figure 1.5).

Drug seeking is inherently instrumental (see Figure 1.5) – the delivery of drugs is typically determined by an individuals' behaviour and these actions become paired with drug delivery. In a series of reviews on the topic Everitt *et al.* suggest that drugs of abuse result in the pathological recruitment of normal learning mechanisms, resulting in aberrantly strong memories (Everitt and Robbins, 2005; Everitt and Robbins, 2016; Everitt *et al.*, 2001). Early studies (e.g. Adams, 1982) demonstrated that instrumental responding undergoes a progression from an initial goal-directed stage, governed by direct associations between an instrumental action and its associated reinforcer to a later, habitual phase, whereby behaviour becomes autonomous (Everitt and Robbins, 2016). Whilst habits in themselves are not problematic and the devolution of behaviours outside of conscious awareness likely carries several evolutionary benefits, in drug addiction this responding becomes maladaptive. These habits also become compulsive and continued to be carried out in the face of adverse circumstances (Everitt and Robbins, 2016; Jonkman *et al.*, 2012a; Jonkman *et al.*, 2012b).

Responding for drugs of abuse is potentiated by the delivery of reward-paired stimuli. The pairings of these cues occurs via normal learning mechanisms, but the increased ability of drugs of abuse to act as USs means they can acquire an overwhelmingly strong ability to control an individual's behaviour (Everitt *et al.*, 2001). Whilst reward-paired cues have been shown to influence responding during both early and late stages of drug seeking, the transition from initial to habitual drug use is likely coupled with the progressive recruitment of distinct neural structures. Preclinical studies have demonstrated that late-stage cocaine-seeking results in the recruitment of specific dorsal striatal structures, with drug seeking habits hypothesised to result in a dominance in behavioural control from the dorsal striatum (Belin and Everitt, 2008; Murray *et al.*, 2012; Vanderschuren *et al.*, 2005, these studies are discussed at length below). Presentation of cocaine paired cues also results in increased activation of dorsal, but not ventral, striatal structures in human cocaine addicts (Garavan *et al.*, 2000), with this activation corresponding to the degree to that these cues trigger craving in these individuals (Volkow *et al.*, 2006). Similarly, compulsive alcohol use is associated with decreased ventral, but increased dorsal striatal activation in response to alcohol paired cues (Vollstädt-Klein *et al.*, 2010).

Addiction related behaviour likely occurs as a result of pavlovian and instrumental memories, and critically, an interaction between these two types of associations (see Figure 1.5). Therapeutic interventions could therefore aim to target these memories, with the view to reducing the ability of these memories to exert control of the behaviour of drug addicts.

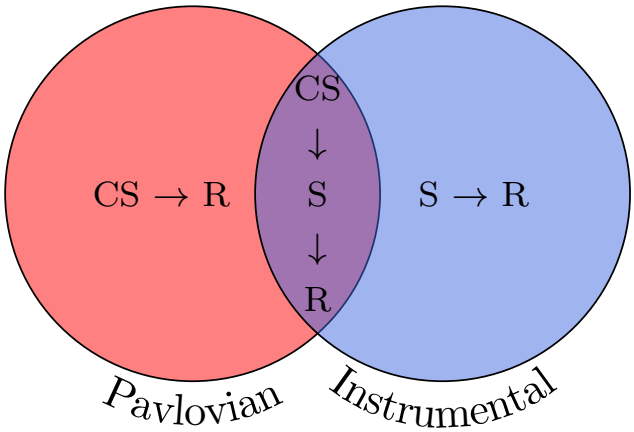


Figure 1.5: Possible memory systems implicated in addiction-related behaviour. CS: conditioned stimulus, R: reinforcer, S: stimulus.

Reconsolidation based treatments

As discussed in previous sections, whilst the reconsolidation literature initially focussed its efforts on aversive associations, more recently it has become apparent that reward-associated memories reconsolidate in a similar fashion. Reconsolidation of associations between discrete drug-paired stimuli and their reinforcers depends upon activation of NMDA (Milton *et al.*, 2008a) and β-adrenergic (Milton *et al.*, 2008b; Schramm *et al.*, 2016) receptors and expression of *zif-268* within the BLA (Lee *et al.*, 2005b; Lee *et al.*, 2006a). Systemic administration of NMDA and β-adrenergic receptor antagonists also disrupts reconsolidation of pavlovian memories formed during drug self-administration (Milton *et al.*, 2008a; Milton *et al.*, 2008b). The contextual stimuli associated with drug use are also hypothesised to contribute to the chronically relapsing nature of drug addiction. These associations have also been shown to undergo reconsolidation that is dependent upon protein synthesis within the BLA, hippocampus and an interaction between these structures (Fuchs *et al.*, 2009; Ramirez *et al.*, 2009). ERK activation within the BLA is also required for reconsolidation of these memories (Wells *et al.*, 2013).

If a similar manipulation could be performed in addicted individuals then the ability of drug paired cues to promote relapse could be reduced, an invaluable therapeutic outcome. Research in human subjects has attempted to prevent memory reconsolidation with the NMDA receptor antagonist memantine, which has been used with some success in the disruption of ethanol-cue reconsolidation in rats (Vengeliene *et al.*, 2015). In Das *et al.* (2015a) individuals attempting to quit smoking were administered this drug prior to a reactivation session consisting of exposure to smoking related cues, alongside being led to believe they would have an opportunity to smoke, only to find out this was not the case, in an attempt to generate PE. However, there was no evidence that memantine prevented memory reconsolidation with these parameters, since subjects reported similar levels of craving regardless of drug administration prior to memory reactivation (Das *et al.*, 2015a). It should be noted that whilst there is some indication memantine may be used to disrupt reconsolidation (Vengeliene *et al.*, 2015), this drug is used as a cognitive enhancer in Alzheimer's disease (Reisberg *et al.*, 2003) and has previously been shown to *enhance* reconsolidation (Samartgis *et al.*, 2012); the effects reported in Das *et al.* may have been the result of a failure of this drug to prevent this process. Alternatively, the results may have been the result of a inability of the memory retrieval session to result in reconsolidation, rather than the pharmacological tool used being unable to prevent it. Determining whether a failure of drug treatment combined with a retrieval session to result

in decreased subsequent memory expression is caused by a failure to induce reconsolidation or an inability to prevent it presents one of the major hurdles for the field.

Since antagonism of β-adrenergic receptors has also shown to be effective in preclinical research (Milton *et al.*, 2008b; Schramm *et al.*, 2016) the use of these drugs to prevent reconsolidation in a clinical population has been explored by Saladin *et al.* (2013). Cocaine addicts underwent a retrieval session consisting of exposure to cocaine cues in the form of video and exposure to simulated cocaine within the laboratory, either followed by a propranolol or placebo pill. The next day, the ability of these same cues to elicit craving was tested. Whilst those administered propranolol did show reduced craving in this test, in contrast to the long lasting effects reported in preclinical studies (Lee *et al.*, 2006a; Monsey *et al.*, 2017), these effects were short lasting and individuals in both treatment groups showed similar levels of craving in a test conducted 1 week later (Saladin *et al.*, 2013).

Whilst preclinical studies have offered much promise in the prevention of reconsolidation with pharmacological agents, only a very small number of studies published to date have investigated whether similar memory processes can be disrupted in individuals suffering from drug addiction. Those that have been conducted have been unable to capitalise on the progress made in the aforementioned preclinical research. Research has also been conducted using memory retrieval trials closely followed with further psychological interventions, rather than pharmacological treatment, in attempts to reduce relapse. In Xue *et al.* (2012) heroin addicts were exposed to a video consisting of cues paired with drug use, not dissimilar to that used by Saladin *et al.* (2013). Rather than following this retrieval session with pharmacological intervention, however, participants underwent a prolonged session of cue-exposure designed to result in extinction. Retrieval followed by extinction resulted in a decreased craving and physiological responses to drug-paired stimuli for at least half a year following the intervention in comparison to subjects that underwent an extinction trial alone or a retrieval trial followed by extinction 6h later, mirroring preclinical research of retrieval-extinction procedures in aversive (Clem and Huganir, 2010; Monfils *et al.*, 2009) and appetitive (Sartor and Aston-Jones, 2014; Xue *et al.*, 2012) tasks. A similar approach has been adopted by Das *et al.* (2015b). Rather than following retrieval sessions with extinction, participants, who were hazardous drinkers, were presented with alcohol paired cues alongside images and tastes designed to trigger disgust in a counter-conditioning session following a retrieval session designed to maximise PE. This treatment resulted in reduced liking and attentional bias toward alcohol paired cues in comparison to control groups that either had similar pairings with non-alcohol associated cues or underwent a reactivation session consisting of no PE. There was, however, no evidence

that the retrieval trial followed by counter-conditioning resulted in a greater decrease in drinking than the counter-conditioning procedure alone (Das *et al.*, 2015b). Thus, whilst attempts to modify memories with non-pharmacological reconsolidation based manipulations have been met with some success (Xue *et al.*, 2012), others have been met with mixed results (Das *et al.*, 2015b). These findings, combined with a paucity of research on the topic, warrants further investigation on the phenomenon.

Food addiction

The prevalence of obesity is increasing (Flegal *et al.*, 2016; Lobstein, 2015). Varying geographically, it is estimated that 38% of individuals in the United States of America (Flegal *et al.*, 2016) and 48% individuals in Europe are overweight, 13% of whom are obese (Gallus *et al.*, 2015). The resulting costs are astronomical; approximately £5bn in the United Kingdom and $150bn in the USA (Lobstein, 2015).

Inarguably an economic burden, whether obesity can be considered a psychological disorder remains a topic of intense debate. The existence of symptoms similar to those observed in drug addiction has been used as an argument for the former view. An extensive review on the topic (Gearhardt *et al.*, 2009a), comparing the (then) 7 criteria of substance dependence (a now obsolete term) as determined by the DSM-IV-TR (APA, 2005) noted numerous similarities in the two behaviours, particularly in the loss of control and repeated failures to reduce intake, with less evidence of tolerance and withdrawal in food addiction. Such similarities have led to the development of the Yale Food Addiction Scale, devised to assist of diagnosis of such eating patterns (Gearhardt *et al.*, 2009b).

Similarities in the neuro-architecture recruited by extensive habitual drug and food-seeking may offer support for the notion that these two behaviours are both similarly reflective of a psychological disorder. Whilst the direction of causality remains a matter for consideration, there is evidence for distinct patterns of neural activation of the striatum in obese and non-obese individuals. Presentation of food-paired stimuli results in increased dorsal striatal activation in obese women *vs.* their lean counterparts (Rothemund *et al.*, 2007), with some evidence suggesting this activity is differentially dependent on the caloric value of the food (cue) presented (Stoeckel *et al.*, 2008). Furthermore, in accord with preclinical studies on rodents (Belin and Everitt, 2008), there is evidence that obesity results in increased connectivity between the dorsal and ventral striatum. The resting state connectivity between these two regions can predict weight gain across a 12-week period

(Contreras-Rodríguez *et al.*, 2017), although it might be argued that increases in the connectivity of these two regions upon exposure to food-paired cues might better demonstrate the potential similarities between drug and food addiction.

Regardless of the neural structures required, the ability of food-paired cues to elicit craving is widely accepted. Meta-analysis on the subject confirms this alongside a resultant increase in eating behaviour, although there was no evidence this relationship was moderated by obesity (Boswell and Kober, 2016). There is some evidence the psychological manner in which these cues varies in lean and obese individuals. Attentional biases to foods, particularly those that are 'unhealthy' (Calitri *et al.*, 2010) can predict increased body-mass index (Yokum *et al.*, 2011). Furthermore, on presentation of food-paired cues restrained eaters show a more specific increase in craving for the food paired with the cue presented, whilst unrestrained eaters show a more diffuse increase in craving (Fedoroff *et al.*, 2003).

A reduction in the impact of these cues to promote food-seeking clearly has potential for the treatment of obesity. Research using reconsolidation based techniques to result in such a decrease is extensive and mechanisms required in this process appear to be preserved between food and drug reward memories. Blockade of reconsolidation with antagonism of NMDA receptors results in decreases in the ability of food-paired cues to support subsequent ANR (Lee and Everitt, 2008a), responses on a previously acquired instrumental behaviour (Flavell and Lee, 2013; Lee and Everitt, 2008b), autoshaping and PIT (Lee and Everitt, 2008c). Treatment with propranolol results in reactivation-dependent impairments in ANR similar to those observed in cocaine self-administration (Milton *et al.*, 2008b). There is, therefore, potential for the incorporation of reconsolidation based treatments into therapeutic interventions for obesity.

Reconsolidation effects: lost in translation

Several psychological disorders lend themselves to possible interventions with reconsolidation based treatments. Despite this, reports of successful interventions using this approach have been limited; whilst attempts to integrate such treatments in the clinic have yielded some success (Brunet *et al.*, 2008; Kindt and Emmerik, 2016; Xue *et al.*, 2012), in numerous other cases these have been unsuccessful in resulting in long lasting decrease in craving (Das *et al.*, 2013; Das *et al.*, 2015a; Saladin *et al.*, 2013; Surís *et al.*, 2013; Wood *et al.*, 2015). As discussed above, retrieval sessions that result in reconsolidation are difficult to detect, only typically evidenced by their induction to result in susceptibility of the memory to amnestic agents. Combined with this, there are several conditions

to be met in order for a retrieval trial to result in reconsolidation. Meeting these criteria will likely be the most difficult hurdle to cross in the development of reconsolidation based treatments for psychological disorders. In order to maximise the utility of preclinical research it must endeavour to model the characteristics of memories underlying psychiatric disorders. This will enable clinical research to anticipate any issues that may arise in the development of retrieval trials that result in memory destabilisation. Here some of the possible issues that may arise in translating the preclinical literature into treatments for the conditions outlined above are discussed, with a particular focus on drug addiction.

Focus on pavlovian associations

The vast majority of reconsolidation studies using self-administration procedures conducted to date have focussed on pavlovian pairings formed during these sessions. Alongside these associations, instrumental memories likely contribute to the maintenance of drug addiction (Everitt *et al.*, 2001). Despite this, instrumental memories, those associating responses and their outcomes (see Figure 1.6A), have received minimal attention in the literature. This is likely to be partly the result of a series of studies by Hernandez *et al.* (2002) showing that nucleus accumbens infusions of anisomycin immediately after instrumental training only result in impairments in later task performance if they occur after early sessions, with no effect reported if infusions occur in later stages of training. It was therefore suggested that these responses undergo a consolidation phase early in training, which was interrupted by the infusions of anisomycin. Once this consolidation phase was complete, these data suggested the memory could not be reactivated and these memories do not undergo reconsolidation.

Whilst many of the studies described in previous sections detailing disruption of reward-related memories required animals to make an instrumental response in the test sessions, the decreases in responding in these sessions occurred as a result of decreased ability of drug-paired cue to potentiate or support this responding. When the CS is removed in test sessions groups respond similarly (and at a lesser rate), regardless of treatment at reactivation (Flavell and Lee, 2013; Lee *et al.*, 2006a). Similarly, when the CS-US association is tested through the ability of the reward-paired stimulus to support ANR, nosepoke responses that were originally paired with drug reinforcement (and are now without consequence) are unaffected by the administration of amnestic agents during memory reactivation (Milton *et al.*, 2008a).

Instrumental associations can be represented by parallel psychological and neural systems, potentially making disruptions of these memories problematic. It has been demonstrated that during the early stages of instrumental training responding is primarily governed by direct associations between the action and the reinforcer. The reliance of this responding upon these associations can be demonstrated through outcome devaluation procedures (see Figure 1.6B and 1.6C). Early studies have shown that after a relatively short period of training animals will decrease their responding in response to reinforcer devaluation (Adams, 1982; Adams and Dickinson, 1981; Colwill and Rescorla, 1985). However, as training progresses, under certain conditions, animals' responding becomes impervious to these devaluation protocols (Adams, 1982). This transition has been described as the progression from responding that is goal-directed, primarily governed by action-outcome (A-O) associations, to one that is habitual, now driven by associations between the environmental stimuli and the responses they have been associated with (S-R responding; Dickinson and Balleine, 1993; Everitt *et al.*, 2001; Wit and Dickinson, 2009).

The progression from A-O to S-R patterns of responding not only recruits distinct psychological processes, but in parallel to the shifts in associative basis of responding is a change in the neural structures responding is dependent upon. The dorsomedial striatum (DMS) appears to be particularly important for the acquisition and retrieval of A-O memories. Pre-training lesions of this structure result in a loss in sensitivity of instrumental responding to devaluation (Yin *et al.*, 2005b). More detailed investigations of the specific role of this structure have shown that it is not only required for the learning of A-O associations (Yin *et al.*, 2005a), but also their expression (Yin *et al.*, 2005b). In contrast, lesions of an adjacent structure, the dorsolateral striatum (DLS), appear to result in an opposite pattern of behavioural deficits, restoring the ability of over-trained animals to express instrumental responding that is sensitive to reinforcer devaluation protocols (Yin *et al.*, 2004).

Double-dissociations in the neural representations of A-O and S-R associations are found in several other brain regions. For example, whilst the prelimbic (PL) cortex appears to be required for goal-directed behaviour, the infralimbic (IL) cortex is required for habitual responding (Coutureau and Killcross, 2003; Coutureau *et al.*, 2009; Killcross and Coutureau, 2003). Lesions of the mediodorsal thalamus also result in impairments in the ability of animals to use A-O based associations to guide responding (Corbit *et al.*, 2003). Finally, whilst the BLA has a demonstrable role in the expression of A-O associations, lesions of the anterior CEN results in an impairment in habit formation (Lingawi and Balleine, 2012). The regions associated with goal-directed and habitual responding likely function in unison via cortico-striatal loops to regulate these two forms of responding. For

example, disconnecting the CEN and DLS with contralateral lesions of these two structures results in deficits in habitual responding akin to bilateral lesions to each of these structures (Lingawi and Balleine, 2012).

The existence of parallel neural structures underlying instrumental associations may pose issues for investigations attempting to disrupt the reconsolidation of these memories. In several of the studies discussed above, lesions of the structures involved in A-O or S-R responding have no net effect on response rates; behavioural deficits are only apparent when tests designed to specifically probe the ability of the animal to recall the association between the instrumental response and its outcome are used. Given that none of the studies discussed previously on the topic of instrumental reconsolidation included tests of this nature, it is possible that the attempts to prevent reconsolidation of these memories led to similar deficits. Specifically, the lack of retrieval-dependent amnestic effects in these studies may have been the result of the use of under sufficiently sensitive tests of the instrumental memory. Reconsolidation of A-O or S-R memories may have been disrupted in these studies but the absence of specific tasks designed to probe these associations may have precluded detection of these effects.

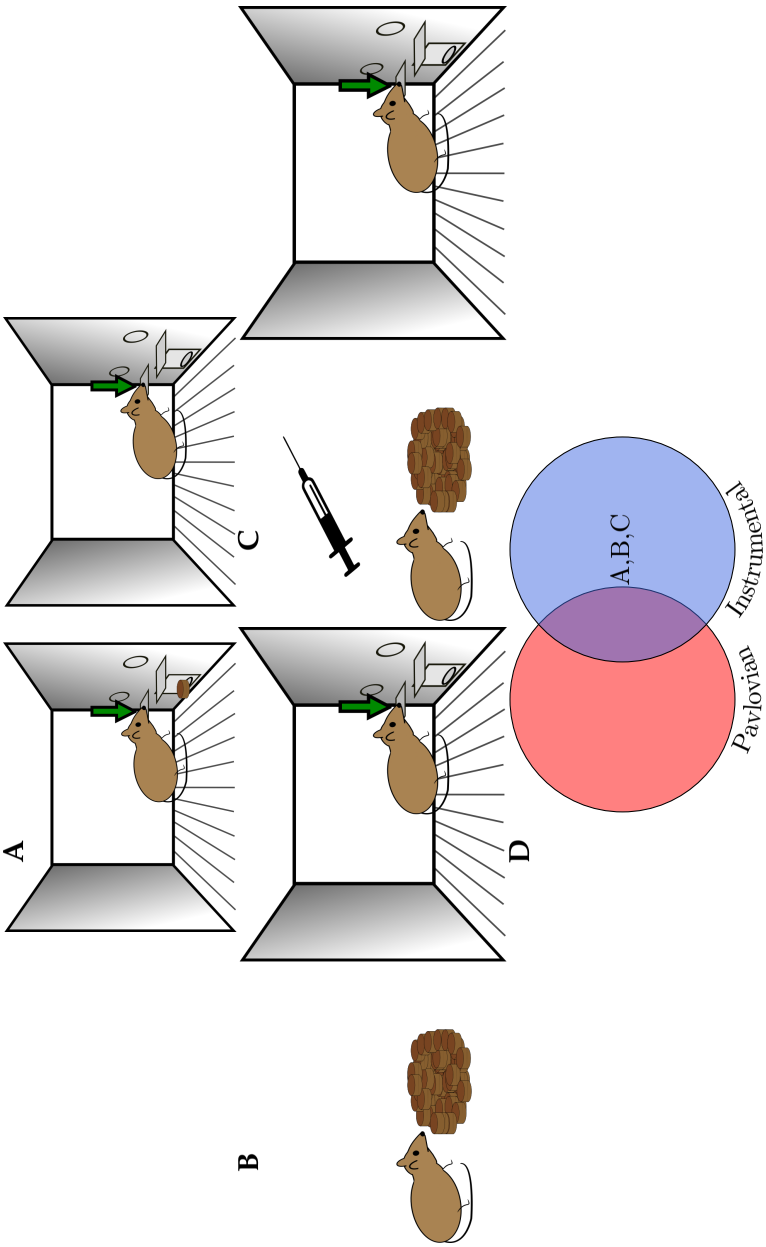


Figure 1.6: Tasks used to study instrumental memories in rodents. In each case, the left hand box shows the procedure used in training and the right used at test. Green arrows represent instrumental responding. **A**: Instrumental responding. Note that reward-delivery is not paired with CS presentation. **B**: Excessive pre-feeding of the reinforcer used in pre-training can be used to probe the ability of animals to retrieve the association between an instrumental action and its reinforcer **C**: Pairing the reinforcer used in training with sickness can act as a similar test. The syringe symbol represents an injection of lithium chloride, resulting in gastric malaise. **D**: Venn diagram showing the suggested memory mechanisms tested with this task.

Despite the apparent difficulties in disrupting instrumental memory reconsolidation, a small number studies on this topic have been published without the addition of reinforcer devaluation protocols. Barak *et al.* (2013) report that reconsolidation of an alcohol self-administration memory can be disrupted by treatment with the mTOR inhibitor rapamycin, either systemically or directly into the CEN. Treatment with this drug results in substantial decreases in alcohol seeking behaviour in later tests when combined with a reactivation session consisting of execution of the once rewarded operant response and the delivery of non-pharmacologically active dose of alcohol at the start of the session (no other alcohol was delivered). Unlike previous studies, the delivery of alcohol in these sessions was not paired with response contingent presentation of any stimuli, suggesting the deficits were the result of impaired instrumental, rather than pavlovian reconsolidation. However, the effects of rapamycin did not appear to depend upon execution of the instrumental response, as would be expected if this were the case, but rather delivery of alcohol in the reactivation session. Access to a similar dose of alcohol used in the operant reactivation sessions in the home cage resulted in reactivation-dependent effect of rapamycin which, unlike the retrieval sessions that consisted of instrumental responding, resulted in a deficit in subsequent alcohol seeking so pronounced that animals no longer distinguished between the levers that did, and did not, previously yield reinforcement (Barak *et al.*, 2013).

One possibility is that associations between the context and the reinforcer were disrupted in the Barak *et al.* (2013) study, rather than the instrumental associations. In this study there was a forced abstinence period between alcohol self-administration and the reactivation session (often and contentiously termed *incubation*). It has been suggested that these periods of forced abstinence promote the ability of reward-associated cues to potentiate responding, without affecting responding in the absence of these cues (Grimm *et al.*, 2001). It is possible, therefore, that this period of abstinence between training and test increased the ability of the reward-associated cues, most likely contextual (given that no discrete cues were paired with reward), to influence responding, and the differences seen in responding were the results of disruptions of pavlovian memories between the context and reward, rather than instrumental associations. Indeed, it is notable that activation of the mTORC1 pathway was localised to the CEN, a region associated with these CS incubation effects (Funk *et al.*, 2006; Li *et al.*, 2015; Lu *et al.*, 2005). It is also perhaps worth noting that it was not explored whether the rapamycin treatment resulted in a conditioned taste aversion (CTA) to alcohol. Previous studies using intra-cranial infusions of anisomycin have shown that such reinforcer devaluation effects can lead to a decrease in responding apparently unrelated to protein synthesis inhibiting properties of this compound (Jonkman and Everitt, 2009; Jonkman and Everitt, 2011). Given that the apparently

amnestic effect in the Barak *et al.* study was determined by exposure to the reinforcer it is possible these results were similarly caused by the generation of a CTA.

Tedesco *et al.* (2014b) have further investigated instrumental memory reconsolidation. After 10d of nicotine self-administration followed by a 14d period of abstinence animals underwent a reactivation session consisting of 20 lever presses in the absence of primary reinforcement. Either 30 minutes before or immediately following this session animals received injections of the NMDA receptor antagonist MK-801. Post, but not pre-reactivation treatment with this drug resulted in a decrease in responding in a test session conducted the next day. Whilst the decrease in responding from non-reactivated controls was relatively small, and perhaps aided by the ability of the reactivation session to increase responding, and MK-801 to prevent this effect, this paper did appear to show disruption of reconsolidation of an instrumental memory. The potential for the decreases in responding being caused by disruptions of context-reward associations were appropriately controlled for with the inclusion of groups exposed to the context without the levers present (Tedesco *et al.*, 2014b).

Only two papers published to date have acknowledged the potential issue of parallel representation A-O and S-R representations of instrumental memories. A series of studies conducted by Exton-McGuinness *et al.* have demonstrated that reconsolidation of both A-O and S-R memories can take place in retrieval sessions consisting of a shift from a predictable fixed ratio (FR) schedule of reinforcement to an unpredictable, variable ratio (VR) schedule. For example, in animals trained to respond for a food reinforcer to an extent where responding was primarily governed by A-O associations, shifting from an FR1 schedule of reinforcement to a VR5 schedule results in reconsolidation that is susceptible to pre-treatment with NMDA receptor antagonism (Exton-McGuinness and Lee, 2015). When animals were given extensive training, now resulting in the dependence of responding on S-R associations, whilst a shift to VR5 schedule of reinforcement was no longer sufficient to result in destabilisation, reactivation sessions conducted under a VR20 schedule of reinforcement resulted in reconsolidation of these memories (Exton-McGuinness *et al.*, 2014). Interestingly, in each case, reactivation sessions conducted in extinction were insufficient to labilise A-O or S-R memories. However these experiments did not include tests of reinforcer devaluation, raising the possibility that these reactivation sessions resulted in A-O or S-R specific memory deficits. Alternatively, these retrieval sessions (that were of a shorter duration than the VR20 reactivation sessions) may have not resulted in sufficient PE to trigger reconsolidation mechanisms. Indeed, for habitual

memories an FR20 reactivation was insufficient to trigger reconsolidation, suggesting that the unpredictable nature of the VR20 sessions was responsible for the destabilisation of the instrumental trace (Exton-McGuinness *et al.*, 2014).

It is important to acknowledge that whilst habitual responding can become divorced from the value of reinforcer it produces (Dickinson and Balleine, 1993; Everitt *et al.*, 2001; Wit and Dickinson, 2009), S-R memories are not necessarily insensitive to reward omission (PE). The key difference between goal-directed and habitual associations is not whether the instrumental association is paired with reinforcement (this is true for both A-O and S-R memories), but rather whether the response is paired with a specific reinforcer. Only goal-directed memories include an association between a response and a specific reinforcer, whilst S-R memories pair instrumental actions with a non-specific, reinforcing outcome. Animals responding habitually are able to reduce their responses in cases of reward omission (Thrailkill and Bouton, 2015) or when instrumental responding leads to delivery of an outcome that is no longer valued (Adams, 1982; Dickinson *et al.*, 1983; Furlong *et al.*, 2014) (in contrast to when these tests are tested in the absence of a reinforcer). If habits were entirely insensitive to reward delivery animals would continue to respond in both of these cases. The sensitivity of habitual memories to reinforcer omission is likely explained by their use of temporal difference rules to guide behaviour (Daw *et al.*, 2005, see Equation 1.1), which take into account the value of the reinforcement obtained *vs.* what is expected, but do not include sensory-specific properties of the outcome.

There is, therefore, a growing literature suggesting that instrumental memories do indeed reconsolidate. However, in some cases this can be achieved with reactivation sessions conducted in the absence of the reinforcer (Tedesco *et al.*, 2014b), whilst in others these non-reinforced reactivation sessions are without effect (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014). While much more research is required to determine the fundamental principles that allow the generation of appropriate and sufficient PE for instrumental memories to destabilise, it appears that inducing the destabilisation of these memories is a practical problem to be solved, rather than a theoretical issue that cannot be overcome. Given that reinforcer delivery may not necessarily be plausible in abstaining individuals (given this may induce reinstatement), and it appears that in some cases it is possible to conduct reactivation sessions without reinforcer delivery, further attempts should be made to delineate the conditions that permit non-reinforced reactivation sessions to trigger destabilisation of A-O and S-R instrumental memories.

Research on the reconsolidation of pavlovian associations must also consider the extent of training

Whilst the extent of training affects psychological processes and neural structures underlying instrumental responding, the number of pairings that pavlovian associations have undergone should also be a critical consideration for research investigating reconsolidation of these memories. In pavlovian fear conditioning, which can be learned with a single CS-shock pairing, strong training occurring as a result of 3 pairings results in a greater resistance of the memory to destabilisation (Suzuki *et al.*, 2004). Whilst in some cases this resistance to destabilisation can be overcome with longer retrieval trials (Suzuki *et al.*, 2004) this is not always effective (Wang *et al.*, 2009). This has led to the suggestion that memory strength may be a 'boundary condition' of reconsolidation and that particularly strong memories no longer reconsolidate.

In the drug self-administration literature, reconsolidation deficits have been observed with large numbers of CS-drug pairings (typically 200-500; see Lee *et al.*, 2006a; Milton *et al.*, 2008a; Milton *et al.*, 2008b; Schramm *et al.*, 2016; Théberge *et al.*, 2010). However, it is not clear how to compare the strength of a memory induced by a single CS-shock pairing and that of a single CS-drug pairing formed during self-administration. Whilst it is possible that the extent of training for these appetitive memories has less of an impact on memory destabilisation than for fear memories, it is also possible that appetitive training procedures have not yet reached sufficient numbers of CS-reinforcer pairings to observe the boundary conditions of reconsolidation that have been reported in the fear literature. Furthermore, whilst 200-500 pairings is a vast improvement on conditioned place preference studies, which typically involve 4-8 drug exposures, this is still significantly lower than the number of pairings that those addicted to drugs are suggested to have undergone throughout their lifetime. It has been estimated that a smoker of two years will have undergone 146,000 such pairings (Das *et al.*, 2015a), almost 300 times greater than the upper limits of the preclinical studies conducted to date. This incongruence between the preclinical studies and the disorder they are attempting to model may hinder extrapolation of these studies to the human population and may explain why attempts to do so have been met with limited success (Das *et al.*, 2015a; Saladin *et al.*, 2013).

The extent of training may not only pose issues as a result of the boundary conditions arising from the increased strength of memories, but also the recruitment of distinct neural and psychological processes as drug use becomes prolonged. The neural basis of reward cue-potentiated responding

differs as a function of the extent of training, likely reflective of a similar transition from goal-directed to habitual drug seeking as is seen in instrumental responding. The shift in the reliance upon distinct striatal circuitry throughout the course of instrumental training is mirrored in a very similar fashion in the brain circuitry underlying response contingent CS-presentation potentiated responding. Local infusion of the dopaminergic receptor antagonist α-flupenthixol into the DMS, a region implicated in goal-directed instrumental responding, results in decreases in cue-dependent cocaine-seeking after relatively short periods of training whilst this procedure is without behavioural effect after extensive training (Murray *et al.*, 2012). In contrast, infusion of α-flupenthixol to the DLS, the adjacent structure implicated in S-R associations results in dramatic decreases in responding governed by response contingent cues after protracted periods of cocaine self-administration (Belin and Everitt, 2008; Murray *et al.*, 2012; Vanderschuren *et al.*, 2005). This treatment has no impact on responding after limited self-administration training (Murray *et al.*, 2012). A similar double disassociation exists between the different subregions within the amygdala: inactivation of the BLA, but not the CEN results in reduced cue-dependent cocaine-seeking after relatively short periods of cocaine access, whilst inactivation of the CEN, but not BLA results in deficits in decreases in well-established cocaine-seeking (Murray *et al.*, 2015).

The distinct neural and psychological processes underlying responding at different points in training has important implications for the research conducted to date investigating the reconsolidation of cocaine-paired memories. It is the responding that occurs after extended periods of drug self-administration that is hypothesised to characterise drug addiction (Everitt and Robbins, 2005; Everitt and Robbins, 2016) – if reconsolidation based treatments are to be of therapeutic value it is the cues that result in S-R responding that must be targeted. As mentioned previously, the majority of studies in cue-cocaine memory reconsolidation conducted to date have involved animals that have received 200-500 pairings during training which is approximately equivalent to the early-stage of cocaine-seeking discussed above. Whether CS-drug associations underlying responding governed by S-R memories reconsolidate is unclear.

It is likely that the neural structures underlying the reconsolidation of these S-R associations are different from those that have been implicated in reconsolidation of CS-drug associations after relatively few days of training. Whilst the majority of studies on CS-drug memory reconsolidation conducted to date have focused on the BLA (e.g. Lee *et al.*, 2005b; Sanchez *et al.*, 2010; Wells *et al.*, 2013), as discussed above, after extended periods of cocaine-seeking inactivation of this structure is without effect, with this responding now dependent upon the CEN (Murray *et al.*, 2015). It is possible that the shift in dependence in responding on these two regions of the amygdala results

in a resistance to destabilisation or recruitment of distinct plasticity mechanisms in mediating this process. The distribution of GluN2A and GluN2B receptor subtypes, which have been shown to have distinct roles in fear memory destabilisation (Milton *et al.*, 2013), differ between the BLA and CEN. Cell firing occurring as a result of NMDA receptor stimulation is more susceptible to GluN2B selective antagonism within the CEN than the BLA (Sah and De Armentia, 2003). Given that the ability of broad spectrum NMDA receptor antagonists to prevent reconsolidation is hypothesised to depend upon the balance of GluN2A and GluN2B subunit activation (Milton *et al.*, 2013), the shift in dependence from the BLA to the CEN may result in a resistance to destabilisation and/or efficacy of these compounds to prevent reconsolidation.

It is important to acknowledge that, as mentioned previously, despite the hypothesised reliance of drug seeking behaviour in human addicts on these habitual patterns of behaviour (Everitt and Robbins, 2005; Everitt and Robbins, 2016; Everitt *et al.*, 2001) and the possible issues that may arise when attempting to target these memories, there has been some success in reconsolidation based treatments in heroin addicted individuals (Xue *et al.*, 2012). This perhaps suggests that the boundary conditions arising from the high number of pairings and habitual response patterns need not be of concern. However, there is little merit in conducting preclinical research if these studies do not truly reflect the disorder they are trying to model.

The Xue *et al.* (2012) study is one of only very few conducted to date able to demonstrate long-lasting decreases in cue-reactivity as a result of the use reconsolidation based treatments; other studies have only been able to demonstrate a transient decrease in cue-induced craving (Saladin *et al.*, 2013). There is a great deal more investigation to be conducted before reconsolidation based treatments are universally adopted. Furthermore, whilst the reliance of retrieval-extinction procedures on reconsolidation based mechanisms was once assumed, several investigations have challenged this notion. For example, the order in which the retrieval and extinction sessions are conducted does not appear to influence the ability of the extinction sessions to result in decreased relapse like behaviour (Millan *et al.*, 2013). One possible reason that the Xue *et al.* paper was not hindered by boundary conditions in drug addicts is that the retrieval-extinction effects do not depend on reconsolidation, and thus the efficacy of these procedures is not affected by memory strength in the same way.

Reconsolidation effects are not always replicable

If reconsolidation based treatments are to provide a plausible therapeutic intervention it is crucial that the experimental data upon which they are based upon are robust and replicable. If the

conditions that trigger reconsolidation of memories cannot be replicated in the highly controlled conditions of laboratory studies (in both animal and human research) there is little hope for the use of similar interventions in individuals suffering with neuropsychiatric disorders. The nature of the associations underlying these conditions is likely to be wide and varied, with patients presenting with maladaptive memories of varying ages, strength and conditions of retrieval that result in sufficient PE to result in destabilisation. However, studies from different laboratories have reported differences in the boundary conditions required to trigger reconsolidation and different pharmacological requirements to prevent reconsolidation. There are a small number of cases of experiments apparently being conducted in very similar ways, in some cases in the same laboratory, yielding different results (Bos *et al.*, 2014; Kindt *et al.*, 2009).

There are several examples of studies reporting that some memories do not reconsolidate, only for later works to be conducted presenting evidence that they do. For example, as discussed above Hernandez *et al.* (2002) suggest that instrumental memories do not reconsolidate, only for a series of studies to be produced a decade later showing these memories can undergo destabilisation and subsequent reconsolidation (Barak *et al.*, 2013; Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014; Tedesco *et al.*, 2014b). Similarly, it was suggested that inhibitory avoidance memories do not reconsolidate (Cammarota *et al.*, 2004), but more recent research shown that they do (Fukushima *et al.*, 2014). Finally, whilst initial reports suggested that goal-tracking memories do not reconsolidate (Blaiss and Janak, 2007), a recent thorough investigation on the topic suggests that through careful manipulation of the training parameters this process can indeed be disrupted (Reichelt and Lee, 2013a). However, these studies do not preclude the use of reconsolidation based treatments for psychiatric disorders. The differences are most likely the result of the early studies failing to satisfy the conditions of retrieval required to result in reconsolidation of these memories, rather than an inherent inconsistency in whether they can reconsolidate. Nonetheless, the issues of retrieval sessions not resulting in memory destabilisation remains a significant issue for the field.

What may pose more of an issue to the potential application of reconsolidation based treatments to the clinic is the apparent differing requirements in the ability of pharmacological agents to prevent reconsolidation. There have been varied reports of the efficacy of β-adrenergic receptor antagonism to prevent this process. These results have been eloquently described as being the result of the use of different methods to assess the reinforcing properties of a reward-paired CS; only memories that are dependent on learning mechanisms within BLA (Burns *et al.*, 1993; Cardinal *et al.*, 2002; Hall *et al.*, 2001b; Parkinson *et al.*, 2000) undergo reconsolidation that is susceptible to propranolol treatment (Lee and Everitt, 2008a). However, recent investigations by Dunbar and Taylor (2016) have

shown that this drug is also unable to prevent reconsolidation when the memory is tested with cue-induced reinstatement of responding, a procedure that is dependent upon the BLA (McLaughlin and See, 2003). Whilst it is possible that these results are reflective of an insufficient understanding of the role of the noradrenergic system in reconsolidation, it is also possible they are indicative of the potential inconsistencies in reconsolidation research. Whilst Tedesco *et al.* (2014b) report that pre, and not post-reactivation treatment with MK-801, is effective at preventing reconsolidation of an instrumental memory, Exton-McGuinness *et al.* (2014) and Exton-McGuinness and Lee (2015) report that pre-reactivation administration of MK-801 is able to prevent reconsolidation of these memories. These differences may be the result of the differing parameters used for reactivation (non-reinforced *vs.* reinforced) or the reward used in training (nicotine *vs.* cocaine and sucrose). However, the apparent differences in training and the resultant shifts in the timing of NMDA receptor dependence mean that it is not entirely clear when the best time point to administer these drugs would be in a clinical setting.

Perhaps most problematic, however, are cases where reconsolidation blockade has been reported with a specified reactivation and pharmacological treatment and subsequent investigations have failed to replicate these effects. A series of studies from the Kindt group have reported that fear memories generated in the laboratory can undergo reconsolidation that is susceptible to treatment with the β-adrenergic receptor antagonist propranolol (e.g. Kindt *et al.*, 2009; Sevenster *et al.*, 2013; Sevenster *et al.*, 2014). However, in a recent study it was reported by the same group that despite using similar training and retrieval conditions as previously used, propranolol was no longer effective in this regard (Bos *et al.*, 2014). Attempts to replicate the amnestic effect of propranolol administered following reactivation carried out by researchers outside of this group have also been unsuccessful (Thome *et al.*, 2016), although it is worth noting that Kindt and colleagues have since once again been able to disrupt reconsolidation with this drug (e.g. Soeter and Kindt, 2015b). Hardwicke *et al.* (2016) have also reported difficulties in replicating reconsolidation experiments. In 2003 a now seminal study was published by Walker *et al.* showing that memories underlying a sequential finger tapping motor task undergo a period of lability following their reactivation that permits interference by new learning. However, extensive recent attempts to replicate this effect have been unsuccessful (Hardwicke *et al.*, 2016). The tendency to not publish failed studies (Coursol and Wagner, 1986; Dickersin, 1990; Ioannidis, 2005, although see Das *et al.*, 2013) likely means that these studies are just the tip of the iceberg with regard to failed replications of reconsolidation research.

Whilst the inability to replicate original studies successfully demonstrating reconsolidation of these memories does not negate the original findings, it is important that future studies do not assume

any previous effects will be replicated. Only those findings that are sufficiently robust such that they can be replicated between laboratories and experimenters are likely to be of use in informing future therapeutic interventions.

Aims of this thesis

There were 3 core aims of this thesis.

Firstly, instrumental reconsolidation was investigated and attempts were made to characterise this process. Specifically, the possibility of disrupting one of the parallel memory traces that underpin these memories was explored, using test sessions that enable any differences in the ability of A-O and S-R memories to reconsolidate to be detected. Few investigations to date have explored the possibility these memories may reconsolidate in parallel. A better knowledge of the updating mechanisms underlying these memories may be informative in the treatment of disorders hypothesised to be characterised by an over-reliance on habitual instrumental memories.

Secondly, the propensity of an appetitive pavlovian memory to reconsolidate after both limited and extended training was investigated. Whilst the impact of extent of training has received some attention in purely pavlovian protocols, few studies have investigated this for memories formed during drug self-administration. Given that associations underlying drug addiction have been formed over a prolonged period of time, an improved understanding of the ability of the memories formed during longer periods of self-administration (resulting in many CS-US associations) to destabilise will provide invaluable insight into treatments for drug addiction encompassing reconsolidation blockade.

Finally, the specific psychological requirements for fear-memory destabilisation to take place and the pharmacological treatments that can prevent reconsolidation of these associations were explored. A better understanding of these processes will not only inform treatments for psychological disorders typified by aversive memories, but also lead to a better understanding of the requirements for reconsolidation of other types of memory to take place.

Because of the potential issues with replication of reconsolidation effects, positive control experiments were always included. This ensured that when training and reactivation parameters were modified from previous reports, the potential loss of the ability of a memory to destabilise could be attributed to these manipulations, rather than a failed replication.

Chapter 2: General methods

Subjects and housing

All subjects were male Lister-Hooded rats (Charles River, Bicester, UK), housed in groups of 4 on arrival and for the duration of experiments that did not require surgical procedures. Animals were housed singly after intravenous surgery or in pairs after intracranial surgery. Animals were housed in a reverse cycle vivarium (lights off at 0700). Food and water were provided *ad libitum* except where stated.

All experimental procedures were conducted in accordance with the Animals and Scientific Procedures Act (1986) and EU Directive 2010/63/EU for animal experiments under project license 70/7548 and personal license number IDD9CA2FC.

Surgery

Anaesthesia

In cases where injectable anaesthesia was used animals were maintained at a surgical plane of anaesthesia using a ketamine ($33\,mg\,kg^{-1}$; Ketaset: Fort Dodge Animal Health, Fort Dodge, IA) and xylazine ($66\,mg\,kg^{-1}$; Rompun: Bayer, Leverkusen, Germany) cocktail administered via the intramuscular (im) route. If required, animals were given an additional injection of ketamine (10 mg, intraperitoneally (ip)) during surgery.

In cases where gas anaesthesia was used this was maintained with inhalation of isoflurane (IsoFlo, Zoetis, London, UK) mixed with 100% oxygen. Induction was achieved with inhalation of 5% isoflurane and maintained at 2-3%, dependent upon the animals' breathing rate. At the end of surgery animals were maintained on 100% oxygen until muscle tone was restored and then transferred to a recovery cabinet.

Intravenous catheterisation

Catheters were obtained from CamCaths (Cambridge, UK) and consisted of a stainless steel cannula within a plastic sheath surrounded by nylon mesh attached to silastic tubing. A plastic bobble was used to secure the catheter in place and the tubing was cut 32mm after this bobble.

Once anaesthetised, an incision was made between the shoulder blades and the right intravenous vein exposed. The catheter tubing was then threaded through the two incisions, such that the port was exposed on the back. The end of the tubing was inserted into the vein and secured in place with suture line (5-0 Mersilk) around the plastic bobble. Both incisions were secured with suture line (5-0/3-0 Mersilk for the front/back) and the animal was left in a recovery cabinet until consciousness was fully regained. Animals were administered oral Baytril (5ml in 500ml drinking water; Bayer Plc, Newbury, UK) for 1d pre and 6d post-surgery. Animals were given at least 7 days to recover before testing began. Catheters were flushed daily with 0.1-0.2ml of heparanized saline 40 units ml^{-1} to ensure and maintain patency.

Intra-BLA cannulation

Immediately following induction of anaesthesia animals were administered the analgesic carprofen (5 mg kg^{-1} subcutaneous (sc); Rimadyl, Pfizer, Kent, UK). Once anaesthetised, animals were placed into the stereotaxic frame (Kopf Instruments), the skull exposed and Epicaine (Dechra Ltd., Stoke-on-Trent, UK) applied to the surgical area. Dorsal-ventral (D-V) measurements were taken for lambda and bregma and a flat skull ensured. 4 jeweller's screws were implanted into the skull, and burr holes drilled at -2.6mm anterior-posterior (A-P) and $\pm$4.5mm medial-lateral (M-L). Guide cannulae (22 gauge, Plastics One) were lowered into the skull via these holes -3.6mm from dura and secured in place with dental cement (Simplex Rapid, Kemdent, Swindon, UK). Obturators (Plastics One, 28G) were inserted into guide cannulae to ensure patency. The surgical wound was sutured (5-0 Mersilk) and the animal placed in a recovery cabinet until consciousness was fully regained. Animals were given at least 7 days to recover before behavioural procedures begun.

Drugs

Systemically administered compounds

(5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) was purchased from Abcam (Cambridge, UK) or Sigma-Aldrich (Dorset, UK), diluted in sterile saline (0.9%) and administered ip at a dose of 0.1 mg kg^{-1}. Details of timing of drug administration are provided in individual chapters. This dose of MK-801 has previously shown to be effective at disrupting reconsolidation of pavlovian aversive (Lee *et al.*, 2006b) and appetitive (Lee and Everitt, 2008a; Milton *et al.*, 2008a) associations and instrumental memories (Exton-McGuinness *et al.*, 2014).

Lithium chloride (LiCl) (Sigma-Aldrich) was administered ip at a concentration of 0.15 M in double-distilled water at a volume of 10 ml kg^{-1} immediately after reinforcer exposure. This was on the lower end of doses previously used to result in a conditioned taste aversion (CTA) (e.g. Tran-Tu-Yen *et al.*, 2009).

(±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) was purchased from Sigma-Aldrich, diluted in sterile saline and administered ip at a dose of 10 mg kg^{-1} 60 minutes before reactivation sessions. This dose of CPP has previously been shown to result in deficits in the consolidation of spatial memories acquired in the Morris water maze (McDonald *et al.*, 2005) and extinction learning (Santini *et al.*, 2001) in rats. This drug has previously been used to prevent reconsolidation in mice (Suzuki *et al.*, 2004).

Rapamycin (LC Labs, MA, USA) was first diluted into a stock solution of 50 mg ml^{-1} in 100% ethanol and stored at −80 °C. The morning of the experiment the solution was brought to room temperature and diluted such that the final vehicle contained 5% ethanol, 4% Peg400 and 4% Tween80, in double distilled water (Blundell *et al.*, 2008; Fifield *et al.*, 2015; Stoica *et al.*, 2011). Injections were administered at a volume of 8 mL kg^{-1} and at a dose of 20 mg kg^{-1}, which has previously been used to prevent reconsolidation of ethanol-paired memories (Barak *et al.*, 2013).

Intravenously administered compounds

Cocaine (MacFarlan Smith, Edinburgh, UK) was diluted in sterile saline to a concentration of 2.5 mg mL^{-1} and the solution microfiltered before use. Fresh cocaine solution was made weekly.

Intracranially administered compounds

Anisomycin (Sigma-Aldrich) was dissolved with hydrochloric acid, pH balanced to 7.4 with sodium hydroxide. The drug was then brought to a final concentration of 125 µg ml^{-1} with phosphate buffered saline (PBS). Aliquots were stored at −80 °C and brought to room temperature on the day of the experiment. Anisomycin was infused at a dose of 62.5 µg side^{-1} in accord with prior studies preventing reconsolidation (Nader *et al.*, 2000).

Injection procedure

Animals that would receive injections ip received a single habituation injection, during which animals rarely showed any signs of distress (sonic vocalisation/ body movement in response to the injection).

Infusion procedure

At least 2h after training sessions animals underwent a mock infusion procedure. Obturators were removed and guide cannulae and replaced with infusion cannulae (28 gauge, 8mm long, projection: 4mm, Plastics One). Animals were then gently restrained for 3.5 min, the injector removed and obturators replaced. For infusions the protocol was similar, except 30s after the injectors had been inserted, anisomycin solution or PBS was infused at a rate of 0.25 µL min^{-1}. Injectors were left in place for a further minute before the obturators were replaced and the animal returned to the home cage.

Histological assessment of cannula placements

At the end of the experiment animals were killed using a rising concentration of carbon dioxide, brains extracted and placed in 4% paraformaldehyde (PFA) solution for at least 48h. Brains were then transferred into a 30% sucrose solution for at least 48h. Brains were sliced on a cryostat at 50 µm, stained with cresyl violet and cannulae tip locations determined under light microscopy. Only animals with cannulae tips located within the basolateral amygdala (BLA) (Paxinos and Watson, 1998) are included in the analysis.

Apparatus

Appetitive experiments

All appetitive experiments took place in Med Associates conditioning chambers (dims: 29.5 x 32.5 x 23.5cm; St Albans, Vermont) conditioning controlled by WhiskerControl (Version 4, Cardinal and Aitken, 2010). The top and two of the sides of these boxes were Perspex, whilst the remaining sides consisted of stainless steel panelling. A houselight (2.8W) was located on one side of the chamber. On the opposite side levers (7cm from the floor) could be presented on the left and/or right hand side of one of the stainless steel sides. Cue-lights (2.5W) were located directly above the levers. Between the levers was a food receptacle that was adapted accordingly, depending on the reinforcer used in the specific experiment. In cases where a liquid reinforcer was used this was delivered via lengths of polythene tubing (1.02mm ID, Portex, Smiths Medical, Kent, UK) into a liquid dispenser comprising of two wells, only one of which was ever used. Where food pellets were used as a reinforcer these were delivered into the magazine via a pellet dispenser. In cocaine self-administration experiments animals were attached to an intravenous tether, allowing solutions to be infused into the catheter whilst the animal was free to move around in the box. Silastic tubing (0.5mm ID, Altec, Durham, UK) was attached to the catheter, protected with a spring sheath and attached a swivel located at the top of the operant chamber. The tubing was attached to a pump (Med Associates), located on the outside of the sound attenuating chamber. Boxes were cleaned with high level laboratory disinfectant (Distel, Tristel, Snailwell, UK).

Food reinforcers

In Chapter 3 reinforcers were either a sucrose-lemon (20% sucrose (w/v; Tate & Lyle, London, UK), 10% lemon squash (v/v; Robinson's, Britvic, Hemel Hempstead, UK)) or maltodextrin-apple & pear (20% maltodextrin (w/v myprotein.co.uk); 10% apple and pear squash (v/v; Robinson's)) solution. In Chapter 4 the reinforcer was always the sucrose-lemon solution. Before training began animals were habituated to each of these reinforcers in half an hour sessions for 4 (Chapter 3) or 2d (Chapter 4).

In the food experiments of Chapter 6 the reinforcer was delivery of chocolate-flavoured reward pellets (AIN-76A, TestDiet, IN, USA).

Fear experiments

Animals in fear experiments were trained in Paul Fray operant chambers (dims: 28 x 28 x 22 cm; Cambridge, UK) located inside sound attenuating shells illuminated by a 1.2W white houselight. Boxes were controlled by WhiskerControl (Version 2, Cardinal and Aitken, 2010). Boxes were equipped with grid floors connected to a shock generator.

In some cases, animals were used in both fear and appetitive experiments. The chambers used in the fear experiments are distinct in size, shape, distance between, and direction of bars of the grid floor, door opening direction than the boxes used in the appetitive experiments. These boxes were also cleaned with a different solution (70% ethanol).

Behavioural procedures

Instrumental memory reconsolidation experiments

Before training animals were food restricted and fed 20g a day of standard laboratory rat chow (SDS, Witham, UK) thereafter.

Training

In Chapters 3 and 4 animals were trained as follows. At the beginning of each session was a two-minute prequel period, where the houselight was illuminated, but the levers were not presented. This period was introduced in an attempt to ensure that animals attended to the environmental stimuli making up the context prior to training, extinction and test sessions in Chapter 3. For consistency this habituation was maintained in Chapter 4. After completion of this period a single lever was presented on the right or left hand side of the magazine (counterbalanced). Reinforcers were delivered on a fixed ratio (FR)1 schedule of reinforcement for the first three days, followed by two days of variable interval (VI)30s. This training procedure was based on similar protocols that have previously been used to result in responding that is sensitive to devaluation with sensory-specific satiety (Nelson and Killcross, 2006). The reinforcer was delivered in 0.1ml aliquots, over approximately 5s. When the reinforcer was delivered no discrete cues were presented, aside from the faint sound of the activation of the pump, located on top of the sound-attenuating shell of the operant chamber. All sessions ended after 62 minutes or when 30 reinforcers had been earned, whichever

came first. Rats that failed to earn at least 20 reinforcers in the final VI30s sessions were excluded from the experiment.

Reactivation sessions

These varied between experiments. Details are provided in the accompanying methods of each chapter.

Reinforcer devaluation

These protocols were only used where training was conducted in the absence of discrete cues.

Sensory-specific satiety

Animals were first placed in the devaluation contexts for 1h, where either the reinforcer used in training or the alternative reinforcer was presented in a spout directly above the magazine.

Pairing with lithium chloride

Sessions began with exposure to the reinforcer used in training in a drinking cage for half an hour, immediately followed by all animals in the Paired group receiving an injection of LiCl. The next day animals were placed in the same cages, for the same duration, but without the reinforcer present. Immediately after these sessions animals in the Unpaired group were injected with LiCl. This cycle was repeated over the next two days (see Figure 2.1A).

Where reinforcer devaluation session took place in the operant chamber the sucrose solution was delivered non-contingently on a variable time (VT)30s schedule for 15 minutes, with the houselight on. Levers were never presented during these sessions. Immediately following the conclusion of this session, animals in the Paired group were given injections of LiCl. The next day, animals were exposed to the operant chamber for the same amount of time as before, but without the reinforcer present. After this session animals in the Unpaired group were injected with LiCl (see Figure 2.1B).

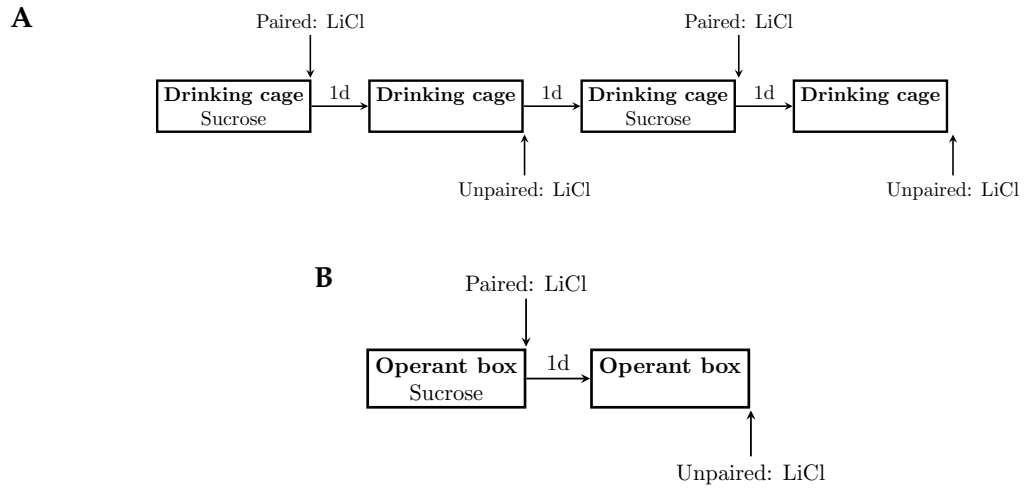


Figure 2.1: Conditioned taste aversion procedure. **A**: Schematic of procedure where devaluation only took place in drinking cages. Animals received injections of LiCl after exposure to either sucrose solution in a drinking cage (Paired group) or the drinking cage alone (Unpaired group). **B**: Schematic of procedure where devaluation took place in operant chambers. After undergoing the same procedures as in A, animals underwent 2 additional days of sucrose-LiCl pairings, except in this case the sucrose was delivered in the operant box, via the magazine that delivered the reinforcer during training.

Test sessions

All instrumental memory test sessions were conducted in the absence of the reinforcer. Further details of the test sessions are provided in the accompanying methods of each chapter.

Cocaine self-administration experiments

Before training animals were food restricted and fed 20g a day of standard laboratory rat chow (SDS) thereafter.

Training

Sessions began with illumination of the houselight and insertion of 2 levers, one of which was designated the active lever (side counterbalanced). Initially, each active lever press (i.e. FR1) resulted in activation of the pump for 5.63s (delivering 0.25mg of cocaine in 0.1ml of saline), and the houselight turning off, both levers retracting and presentation of a light conditioned stimulus (CS) for 20s. Responding on the inactive lever had no consequence, but was recorded as a general measure of locomotor activity. Sessions ended after 2 hours or when 30 reinforcements had been earned, whichever came first.

In experiments where a second-order schedule of reinforcement was introduced animals were initially trained on an FR1 schedule of reinforcement, as above. Fixed interval (FI) schedules of reinforcement were then introduced in the following order: FI1(min), FI2, FI4, FI8, FI10 (1d each) before stabilising for three days at FI15. All sessions ended after 2 hours or when 30 reinforcements had been earned.

In second-order training sessions animals continued to respond on an FI15 schedule of reinforcement, except that superimposed on top of this was a FR10:S schedule, whereby each 10th lever press was reinforced with a 1s CS presentation (this schedule is referred to as FI15(FR10:S)). Both the FI15 and the FR10:S schedule had to be completed in order to earn a cocaine infusion (Arroyo *et al.*, 1998; Everitt and Robbins, 2000). These second-order sessions ended after 5 reinforcements had been earned or 2 hours had passed, whichever came first.

Reactivation sessions

Details of reactivation procedures are provided in the individual methods sections.

Test sessions

Animals that had been trained on FR1 schedules of reinforcement were tested in a *relapse* procedure (Lee *et al.*, 2006a; Milton *et al.*, 2008a; Murray *et al.*, 2012). Active lever presses during hour long these sessions resulted in a brief (1s) presentation of the cocaine paired stimulus but had no other consequences. Additional test sessions of specific experiments are described in the methods section of the accompanying chapter.

Animals that had been trained on FI or second-order schedules of reinforcement were tested under a FI15(FR10:S) schedule of reinforcement (including reinforcer delivery after completion of the first interval).

Food second-order experiments

Experiments were conducted in a similar fashion to the cocaine self-administration experiments using second-order schedules of reinforcement as above. Before training animals were food restricted and fed 18-20g a day of standard laboratory rat chow (SDS) thereafter.

Training

Animals first underwent two sessions of magazine training. During these 30-minute sessions a single pellet was delivered alongside illumination of the CS light and de-illumination of the house-light for 20s. Pellets were delivered on a VT60s schedule. Instrumental training began the following day. In these sessions two levers were presented, responses on one of which resulted in chocolate pellet delivery on the given schedule of reinforcement. As in magazine training, reinforcer delivery was paired with illumination of the CS light and de-illumination of the houselight. The levers also retracted during CS presentation. Following this an FI schedule of reinforcement was introduced, progressing through FI1 (min), 2, 4, 6, 8, 10 and stabilising for FI15 for a final three days. As the interval increased, so did the number of pellets delivered, with a total of 20 pellets being delivered in the FI15 sessions. All sessions finished after 120 minutes or when 40 pellets had been earned, whichever came first. Procedures were adapted from Giuliano *et al.* (2012).

Following pre-training sessions a second-order schedule of reinforcement was introduced: a second FR schedule was superimposed on top of the FI15 schedule, such that each 10th lever press resulted in a brief (1s) presentation of the CS (FI15(FR10:S)).

Reactivation sessions

Details of reactivation procedures are provided in the individual methods sections.

Test sessions

Animals underwent two test sessions, the first of which was conducted the day after the (final) reactivation session. Test sessions were conducted exactly as the second-order training sessions, with the pellets being delivered into the magazine at the end of each interval.

Fear experiments

Training

Animals were first habituated to the operant chambers in 2h sessions. The next day animals were exposed the same box for a further 25 minutes, after which time the CS was presented (clicker, 10 Hz, 80 dB or illumination of a CS light and simultaneous extinction of the houselight, both 60s)

which co-terminated with a single footshock (0.5s, 0.5 mA). After a 5-minute inter-shock interval (ISI) the CS was again presented, co-terminating with a shock. After a further 5 minutes the session ended, the houselight was extinguished and the animal removed from the chamber. The training procedure is presented in schematic form in Figure 2.2. In cases where a single CS-unconditioned stimulus (US) pairing was presented the session ended after the first ISI. Protocols were adapted from Merlo *et al.* (2014).

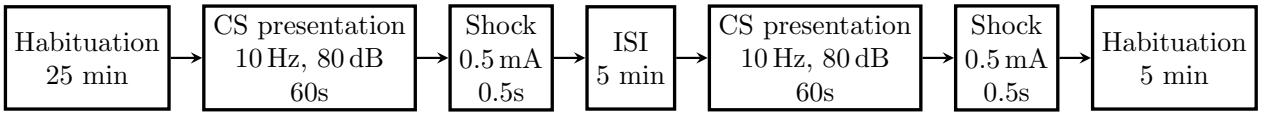


Figure 2.2: Schematic of the auditory fear training procedure.

Test and reactivation sessions

For reactivation and test sessions the CS was presented for 60s after a 1-minute habituation period, with the session terminating 1 minute after presentation of the CS.

Scoring

Freezing behaviour was defined as the absence of movement in the except for breathing and was scored manually offline, with the observer blind to the drug treatment. The total time spent freezing was scored (and converted into a percentage) during all CS presentations in the training session. Freezing behaviour in the reactivation and test sessions was scored during the minute before CS presentation and during the 1-minute CS.

Statistical analysis

In appetitive experiments sessions frequently ended when the maximum number of reinforcements had been earned, rather than when the time limit had elapsed. Because of this, reporting total number of lever presses made in training sessions, particularly those conducted in an FR1 schedule of reinforcement, provides little insight into actual response rates. Responses in training sessions have therefore been transformed to responses per minute (RPM).

Analysis of all experimental data was achieved using analyses of variance (ANOVAs) or t-tests. Variables were coded as between-subjects or within-subjects as appropriate. In cases of a significant interaction between two factors the effect of the first factor was examined in each of the conditions

of the second. In cases of a three-way interaction, the existence of the interaction upon separate analyses of the first and second factors was analysed in the each of the separate conditions of the third. In some cases, where there was a hypothesis to suggest that there should be a differential effect between groups, separate analyses of the different treatment conditions were carried out (Cardinal and Aitken, 2013).

If analysis required comparison of only two conditions this was achieved with t-tests (two-tailed; paired or unpaired, as appropriate). If Levene's test of equality of variance was significant for unpaired t-tests equal variances were not assumed. In cases where there are more than 2 conditions (between or within-subjects) the Šídák correction for multiple comparisons has been applied.

In cases where Mauchly's test of sphericity was significant the Huynh-Feldt (H-F) correction was used when the sum of H-F and Greenhouse-Geisser (G-G) epsilons was greater than 1.5 and G-G used where it was not (Cardinal and Aitken, 2013). In cases where the degrees of freedom have been adjusted this is represented in the text by reporting these values to one decimal point.

Power analysis was conducted with G*Power (V3.0, Faul *et al.*, 2007). In cases where achieved power was approximated from the graphs of published reports this was carried out using Inkscape's measurement tool (V0.91). For analysis of experimental data the required and achieved power was calculated for a significant t-test for what was deemed the most important comparison, without a correction for multiple comparisons applied. This approach overestimates the power obtained, but underestimates the number of subjects required to obtain sufficient power. Given that this analysis was typically conducted in order to ascertain whether a null result was due to low power this approach was deemed most appropriate.

For all the analyses described above α was 0.05.

Chapter 3: Investigations in context-induced renewal: effects of NMDA receptor antagonism at memory reactivation on subsequent goal-directed responding

Introduction

The storage and retrieval of memories is an essential function for everyday life. Early theories suggested that memories undergo a period of vulnerability, or lability, just following their formation, termed *consolidation*. Following this initial period, the consolidation account suggests that memories remain stable indefinitely (McGaugh, 1966; McGaugh, 2000). This view has recently been challenged, with evidence suggesting that memories undergo further transient periods of vulnerability and are reconsolidated following their recall (Nader *et al.*, 2000; Przybyslawski and Sara, 1997). Initially investigated in aversive memories, it is becoming increasingly apparent that appetitive memories undergo a similar period of lability following their reactivation.

Infusion of *zif-268* antisense oligodeoxynucleotides (ASOs) in the basolateral amygdala (BLA) following reactivation of a pavlovian association between a drug and a discrete stimulus results in deficits in the subsequent rewarding properties of the conditioned stimulus (CS) (Lee *et al.*, 2005b; Lee *et al.*, 2006a). Reconsolidation of these CS-drug memories can also be prevented by systemic administration of β-adrenergic (Milton *et al.*, 2008b; Schramm *et al.*, 2016) or N-methyl-D-aspartate (NMDA) receptor (Milton *et al.*, 2008a) antagonists. The reconsolidation of food-paired memories is similarly dependent on the activation of β-adrenergic and NMDA receptors (e.g. Lee and Everitt, 2008a; Milton *et al.*, 2008b). The ability to disrupt such associations has been suggested to be a potential future treatment avenue for conditions hypothesised to be maintained by presentation of these reward-paired cues, most notably drug addiction (Everitt, 2014; Milton and Everitt, 2012) and perhaps obesity (Epstein and Wrotniak, 2010; Reichelt and Lee, 2013b).

Alongside discrete stimuli, contextual cues associated with reward also contribute towards responding and are hypothesised to be a major factor in promoting relapse in abstinent individuals (Fuchs *et al.*, 2008). Whilst associations of this nature are often investigated with conditioned-place preference studies (but see Ito *et al.*, 2002), these tasks bear little resemblance to drug addiction, where drug-delivery is determined by an individual's behaviour and involves many pairings between the context and reinforcer, two characteristics absent from conditioned-place preference protocols. Partly with this in mind, research has investigated the potential role of contextual stimuli to promote relapse-like behaviour using the phenomenon of context-induced renewal (CIR). Here, returning animals to a context in which self-administration has previously taken place after a period of instrumental extinction in a second context results in increased responding. The associations underlying this renewed responding have been shown to undergo reconsolidation that is dependent upon protein synthesis in the BLA, but not dorsolateral striatum (DLS) or dorsal hippocampus, although activation of this latter structure is required for reconsolidation of these associations (Fuchs *et al.*, 2009; Ramirez *et al.*, 2009). Further investigations into the specific molecular requirements of reconsolidation underlying renewed responding have revealed it to be dependent upon extracellular signal-regulated kinase (ERK) (Wells *et al.*, 2013) and protein kinase A (PKA) within the BLA (Arguello *et al.*, 2014).

The majority of reconsolidation studies published to date have investigated responding after training that would be expected to result in responding that is goal-directed. However, the resulting effects on the expression of these action-outcome (A-O) associations has received little, if any, attention in the literature. This is despite an expanding body of evidence demonstrating these two types of memories are subserved by distinct cortico-striatal circuitries. Whilst the dorsomedial striatum (DMS) and prelimbic (PL) cortex are required for goal-directed responding, the DLS and infralimbic (IL) cortex are required for stimulus-response (S-R) memory expression (Coutureau and Killcross, 2003; Killcross and Coutureau, 2003; Yin *et al.*, 2006; Yin *et al.*, 2005b). The parallel circuitries recruited by these different types of instrumental responding raise the possibility that disruptions of reconsolidation could selectively target A-O or S-R memories.

The lack of research on the effects of disrupting reconsolidation on A-O and S-R processes is primarily because research on pavlovian, rather than instrumental, associations has dominated the field. For example, responding in tests of conditioned reinforcement is inherently instrumental – an animals' behaviour impacts upon the presentation of the reward-paired cue. However, responding governed by reward-paired cues does not appear to undergo a transition from being sensitive, to insensitive to devaluation. Whilst lever-pressing that results in the delivery of a food-paired cue is

not decreased by pairing of the associated food with lithium chloride (LiCl) (Parkinson *et al.*, 2005), food cup approaches elicited by presentation of the CS do (Morrison *et al.*, 2015). This has led to the suggestion that the susceptibility of responses to pavlovian stimuli depends upon the proximity of the response to consumption of the reinforcer (Galarce *et al.*, 2007) rather than the extent of training. This makes investigation of the effects of disruption A-O and S-R associations in tasks of this nature difficult to investigate.

In contrast, responding that occurs in CIR has been shown to undergo a transition from being goal-directed to habitual. Responses of this nature are susceptible to devaluation after limited (Cohen-Hatton and Honey, 2013) but not extended training (Thrailkill and Bouton, 2015). CIR can be observed under conditions in which the pavlovian conditioning histories of the training and extinction contexts are equated (Cohen-Hatton and Honey, 2013; Todd, 2013) or similar (Nakajima *et al.*, 2002) and extensive experience of the renewal context in the absence of a reinforcer without the opportunity to make a response does not affect the magnitude of the renewal effect (Bouton *et al.*, 2011). These data, alongside others, suggest that CIR is reflective of context-dependent instrumental response patterns, rather than pavlovian associations akin to those seen in conditioned-reinforcement tasks.

Whilst much debated, the most parsimonious explanation of the renewal effect is that this occurs as a result of the loss of inhibitory instrumental associations that have been formed with the extinction context (Todd *et al.*, 2014). This explains, for example, how renewal can occur when training, extinction and test sessions are conducted in distinct contexts (ABC renewal; Bouton *et al.*, 2011) – the loss of the inhibitory associations formed in B when placed in C result in an increased responding.

Responding in CIR not only undergoes a transition between being sensitive to being insensitive to devaluation (Thrailkill and Bouton, 2015) but is also susceptible to disruptions of reconsolidation (e.g. Fuchs *et al.*, 2009). The experiments in this chapter therefore sought to investigate how disrupting reconsolidation of memories resulting in renewed responding affect the underlying goal-directed associations.

One possibility, rather than using CIR as a tool to characterise instrumental memory reconsolidation, would be to investigate context-independent responses. Several recent investigations have demonstrated that these memories undergo reconsolidation (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014; Tedesco *et al.*, 2014b). However, there appears to be some degree of inconsistency in when amnestic agents need to be administered in order to prevent instrumental

memory reconsolidation. Some reports suggest that NMDA receptor antagonists need be administered before reactivation sessions (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014), whilst others suggesting this treatment is ineffective and only drug-administration following reactivation can prevent reconsolidation of these memories (Tedesco *et al.*, 2014b). Research from Exton-McGuinness *et al.* has also suggested that reinforcer delivery is required in order to destabilise these memories (see also Barak *et al.*, 2013), whilst this is not the case when tests of CIR are used (e.g. Fuchs *et al.*, 2009). Retrieval sessions that result in memory reconsolidation in the absence of reinforcer delivery are likely to be of maximal clinical utility. Retrieval sessions that result in memory reconsolidation without the requirement for reinforcer delivery are likely to be of maximal clinical utility.

The ability to use A-O associations to guide responding can be assessed with reinforcer devaluation, typically achieved through pairing of the reinforcer with sickness or excessive pre-feeding before responding is assessed (e.g. Adams, 1982; Balleine and Dickinson, 1998). Owing to difficulties in the use of similar techniques in animals self-administering intravenously delivered psychostimulants, demonstrating such devaluation effects for drug-seeking has been problematic. Previous studies have used oral cocaine delivery and subsequently devalued this reinforcer in order to investigate the extent that cocaine taking is goal-directed through pairing of the cocaine solution with LiCl induced malaise and subsequent instrumental memory tests (Miles *et al.*, 2003). However, the large number of groups required for these experiments to demonstrate that responding is motivated by delivery of cocaine and not the additional sucrose and flavourings added to the cocaine solution, combined with the necessary between-subjects design for LiCl devaluation make this approach infeasible for reconsolidation experiments. With this in mind, a food reinforcer was used in the experiments described herein. Whilst pairing reinforcers with lithium-chloride induced nausea results in a near-permanent aversion, reinforcer devaluation with sensory-specific satiety is temporary. The use of this latter technique therefore permits the use of a within-subjects devaluation procedure and the continued use of subjects following reinforcer devaluation and was therefore adopted for the experiments described in this chapter.

Once responding in CIR was shown to be sensitive to devaluation after limited training, an experiment was conducted to investigate the reconsolidation of associations underlying expression of goal-directed CIR using the NMDA receptor antagonist MK-801. This drug was chosen on its ability to prevent memory reconsolidation in aversive (e.g. Lee *et al.*, 2006b) and pavlovian appetitive memories (e.g. Lee and Everitt, 2008a). Prior investigations have also shown this drug to be effective at preventing instrumental memory reconsolidation (Exton-McGuinness and Lee, 2015;

Exton-McGuinness *et al.*, 2014). It has not previously been investigated whether reconsolidation of memories underlying CIR requires NMDA receptor activation (although see Wouda *et al.*, 2010).

To summarise the above, experiments in this chapter investigated context-induced renewal in animals trained to respond for a food reinforcer. It was first investigated whether these responses are goal-directed after limited periods of training (Experiment 1). This was assessed with sensory-specific satiety, a manipulation that should result in a decrease in goal-directed, but not habitual responding. Following this, the dependence of the reconsolidation of the memories underlying these responses on NMDA receptor activation was investigated (Experiment 2). These results not only provide insight into the associative structure underlying responding in CIR, but also how these responses are reconsolidated and how disrupting this process might specifically affect A-O or S-R associations. A better understanding of the mnemonic processes underlying these two types of responding may provide insight into future treatments for psychiatric disorders characterised by an imbalance between these two associations.

Methods

Summary

In Experiment 1 animals were trained to lever press in operant conditioning chambers. The response was then extinguished in a novel context across 4 days. Over the course of the following 2 days the reinforcer used in training and a similarly preferred reinforcer was devalued before test sessions, either conducted in the training or extinction context. This allowed a between-subjects comparison of whether CIR was occurring, and a within-subject comparison to determine whether this responding was sensitive to reinforcer devaluation.

Experiment 2 was conducted as Experiment 1, except that a 5-minute memory reactivation session, conducted in the absence of the reinforcer, was interposed between the training and extinction phases. Before this session one third of the animals were injected with the NMDA receptor antagonist MK-801 in order to investigate how this treatment affects the subsequent expression of goal-directed CIR, with the prediction that this may prevent reconsolidation of the memory, leading to a decrease in responding during the test session.

Procedures were conducted as in General methods except where stated.

Subjects

Subjects were 48 male lister-hooded rats weighing 220-370g at the beginning of experiments. The day before reinforcer habituation animals were food-restricted and fed 20g of rat chow at the end of each day."

Apparatus

Chambers were modified so as to form 3 separate contexts (see Table 3.1). Contexts 1, 2 and 3 served as training, extinction and devaluation contexts in a counterbalanced fashion.

Context	Floor	Walls	Auditory
1	Acrylic	Polkadot	Continuous tone (2.5kHz, 74dB)
2	Mesh	Clear	Metronome (120BPM, 65dB,)
3	Grid	Stripes	2s beeping (1kHz, 66dB) / 2s white noise (66dB)

Table 3.1: Additional contextual cues used for boxes in this chapter.

Behavioural procedures

Training

Animals were trained to lever press for either a sucrose-lemon or maltodextrin-apple & pear solution. Before training began all animals were individually habituated to both reinforcers for 30 minutes per reinforcer over 4d in order to reduce neophobia to the solutions in the training and sensory-specific satiety sessions.

Following habituation to the reinforcers animals were trained to lever press for either the sucrose or maltodextrin solution. Reinforcers were delivered on a fixed ratio (FR)1 schedule of reinforcement for the first three days, followed by two days of variable interval (VI)30s.

Approximately two hours after the two VI30s sessions animals were habituated to a third context, which would later serve as a devaluation context. No levers were ever presented in this context, but a spout containing either the reinforcer used in training or the alternative reinforcer was presented directly above the magazine. These sessions were 30 minutes in duration and were conducted to habituate the animals to the devaluation procedure, context and reinforcers.

Reactivation: Experiment 2

Reactivation sessions were conducted the day after the conclusion of training and were a total of 7 minutes in duration. For these sessions the lever was presented two minutes after illumination of the houselight and lever presses resulted in the activation of the pump on the same VI30s schedule as in training, but no reinforcers were delivered. 30 minutes before this session 8 animals were injected with MK-801 (0.1 mg kg^{-1}, Sigma-Aldrich, UK, intraperitoneally (ip)) and the remaining animals were injected with a vehicle solution (saline, 0.9%).

Extinction

Extinction sessions were conducted the day after the conclusion of training (Experiment 1) or the reactivation session (Experiment 2). These took place in a different operant chamber from that used in training and was configured to form a distinct context (see Table 3.1). As in training, sessions begun with a 2-minute prequel period and following this a lever was presented on the same side as used in training. Responding was without consequence and sessions ended after 122 minutes.

Test

Test sessions were conducted the day after the final extinction sessions. Animals were first given the opportunity to consume either the reinforcer used in training or an alternative reinforcer for 1h. Approximately 5 minutes after the conclusion of this session, animals were either returned to their training or extinction contexts, where the lever was presented for 5 minutes. During test sessions responses were recorded but were without consequence. The next day the test was repeated in the same fashion, except the opposite reinforcer to that used in the first test was devalued (order counterbalanced). For Experiment 1 there were two groups in these sessions – one group of rats were always tested in the training context, whilst the second were always tested in the extinction context.

In Experiment 2 animals given vehicle injections at reactivation were split in a similar fashion as in Experiment 1 but all animals given MK-801 were tested in the training context. Because this test failed to reveal any significant differences, several further tests were conducted. First, animals underwent 2 test sessions in the training and extinction context on consecutive days (order counterbalanced) without having undergone reinforcer devaluation beforehand. These were 122 minutes

in duration and were conducted without the reinforcer present. Animals then underwent 2 re-training sessions (in the training context) and two further devaluation tests, each in the training context but were otherwise conducted as before. Between each of these devaluation tests was a single reacquisition session. All reinforced sessions were rewarded on a VI30s schedule.

Statistical analysis

Results from test sessions in Experiment 1 were analysed with a mixed-design analysis of variance (ANOVA), with Devaluation (Training and Alternative reinforcer) as a within-subjects factor and Context (Training or Extinction) as a between-subjects factor. Data from the first test session from Experiment 2 were analysed in a similar way, except the between-subjects factor was Group, which comprised of 3 conditions; the within-subject effect of Devaluation was analysed as before. For the analysis of subsequent test sessions animals were grouped only according to their drug-treatment at reactivation as a between-subjects factor (Drug) with the within-subjects factors from these tests coded accordingly (Context for the 2nd test and Devaluation for the 3rd). Responses during training are presented as responses per minute (RPM) whilst reactivation sessions are reported as total number of lever presses. This is in order to facilitate comparison of data between this and subsequent chapters.

Results

Experiment 1: Context induced renewal is goal-directed after limited-training

Test

Experiment 1 investigated whether responding CIR was sensitive to devaluation after limited training (see Figure 3.1A).

After limited-training animals showed a renewal effect – those returned to the extinction context made fewer responses than those returned to the training context. This renewed responding was sensitive to the current value of the reinforcer used in training (Figure 3.1B). These results were indicated by a main effect of Context ($F_{1,17} = 22.36$, $p < .001$), Devaluation ($F_{1,17} = 19.06$, $p < .001$) and a significant interaction between these factors ($F_{1,17} = 13.65$, $p = .002$). Further analysis of this effect revealed that animals decreased responding in response to devaluation in the training, but not extinction context (Figure 3.1B).

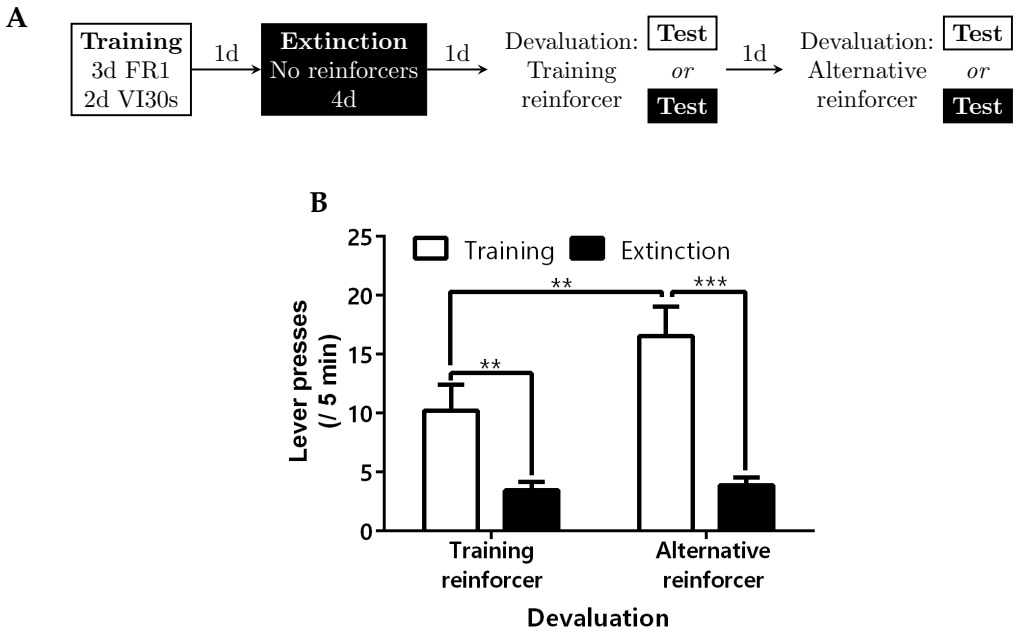


Figure 3.1: Context-induced renewal is goal-directed after limited-training. **A**: Animals were trained for 5d in one context, followed by extinction in a second. Animals were then returned to either the training or extinction context after devaluation of either the reinforcer used in training or a alternative reinforcer. See text for details. **B**: Results of the test sessions. Animals respond more in the training context than the extinction context. Renewed responding is sensitive to devaluation of the reinforcer used in training. Bars represent means +SEM. N=9/10. ** p<.01; *** p<.001

Training and extinction

5 animals were excluded after failing to reach the training criterion. None of the differences occurring between the groups could be explained by differential performance during training or extinction sessions. Prospective groupings did not affect the total number of responses made in the training sessions ($F_{1,17} = 2.01$, $p = .174$) nor the rate of acquisition (Day*Group: $F_{2.1,35.5} = 1.37$, $p = .267$; Figure 3.2A). Prospective groups responded similarly in the extinction sessions ($F_{1,17} = 0.27$, $p = .870$) and reduced responding similarly across days of extinction (Day*Group: $F_{1.5,26.0} = 1.10$, $p = .356$; Figure 3.2B).

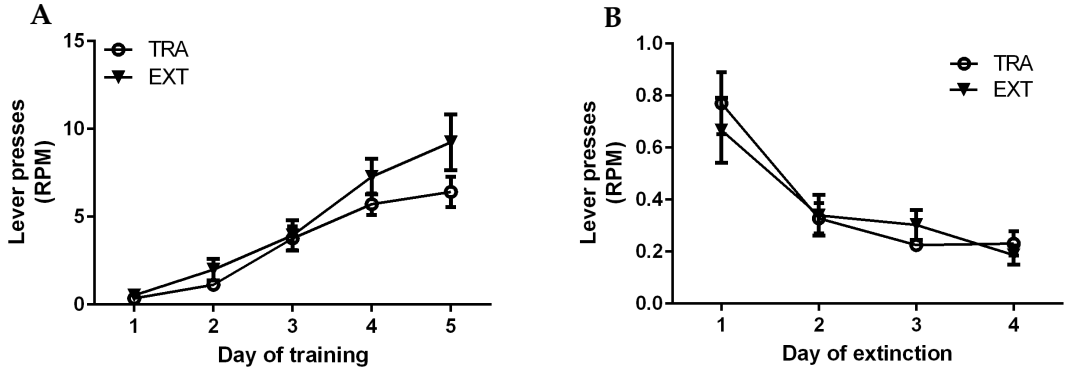


Figure 3.2: Training and extinction data from Experiment 1. **A**: Responding during training. **B**: Responding during the extinction sessions. Legend refers to the context animals would subsequently be tested in. Values represent means ±SEM. N=9/10 per group.

Experiment 2: NMDA receptor antagonism at instrumental memory reactivation does not affect renewal, but prevents expression of goal-directed responding

Test

Neither drug treatment, devaluation nor testing context had a significant effect on the level of responding in the test session (Figure 3.3). This was indicated by an absence of a main effect of Group ($F_{1,21} = 1.34$, $p = .284$), no effect of Devaluation ($F_{1,21} < 0.00$, $p > .999$) and no interaction between these factors ($F_{2,21} = 0.80$, $p = .464$).

In order to further investigate the effect of contextual stimuli on responding in these animals they underwent a second, longer, test in each context, without having undergone reinforcer devaluation beforehand. Data from the two vehicle groups were pooled for this analysis. Animals responded more in the training than the extinction context in this test ($F_{1,22} = 14.38$, $p = .001$), with the effect of context not being affected by drug treatment at reactivation (Context*Drug: $F_{1,22} = 1.45$, $p = .241$; Figure 3.3D).

After the second test, animals were retrained (in the training context) and underwent further devaluation sessions, as conducted before, except that all tests took place in the training context. Although overall responses in this session were not affected by devaluation ($F_{1,22} = 1.64$, $p = .214$), there was a trend towards differential devaluation effect, dependent on the drug given at reactivation (Drug*Reactivation: $F_{1,22} = 3.27$, $p = .084$). When analysed separately, it was revealed that

whilst animals given vehicle at reactivation decreased their responding as a result of reinforcer devaluation ($t_{15} = 2.44$, $p = .028$), this was not true of animals administered with MK-801 ($t_7 = 0.43$, $p = .678$; Figure 3.3E).

MK-801 did not appear to prevent reconsolidation of the contextual associations underlying instrumental responding, as indicated by responding in a second test of context-induced renewal being unaffected by drug-treatment at reactivation. NMDA receptor antagonism with MK-801 did, however, appear to prevent expression of goal-directed responding in a devaluation test after retraining.

Reactivation, training and extinction

The total number of responses made in the training sessions was not affected by prospective groupings ($F_{1,21} = 0.34$, $p = .717$), with all groups increasing lever pressing across training sessions ($F_{1.9,40.5} = 68.30$, $p < .001$) equally (Day*Group: $F_{3.9,40.5} = 0.61$, $p = .768$; Figure 3.4A). The total number of responses made in the reactivation session was not affected by the drug administered prior to the session, nor the prospective context at test (main effect of Group: $F_{2,21} = 2.51$, $p = .106$; Figure 3.4B). Drug treatment or prospective groups also did not affect the total number of responses made in the extinction sessions in the novel context ($F_{1,21} = 0.51$, $p = .606$), with all groups extinguishing ($F_{3,63} = 39.49$, $p < .001$) at similar rate (Group*Session: $F_{3,63} = 0.70$, $p = .650$; Figure 3.4C). This suggested that the context-independent instrumental response was not affected by NMDA receptor antagonism with MK-801 during memory retrieval.

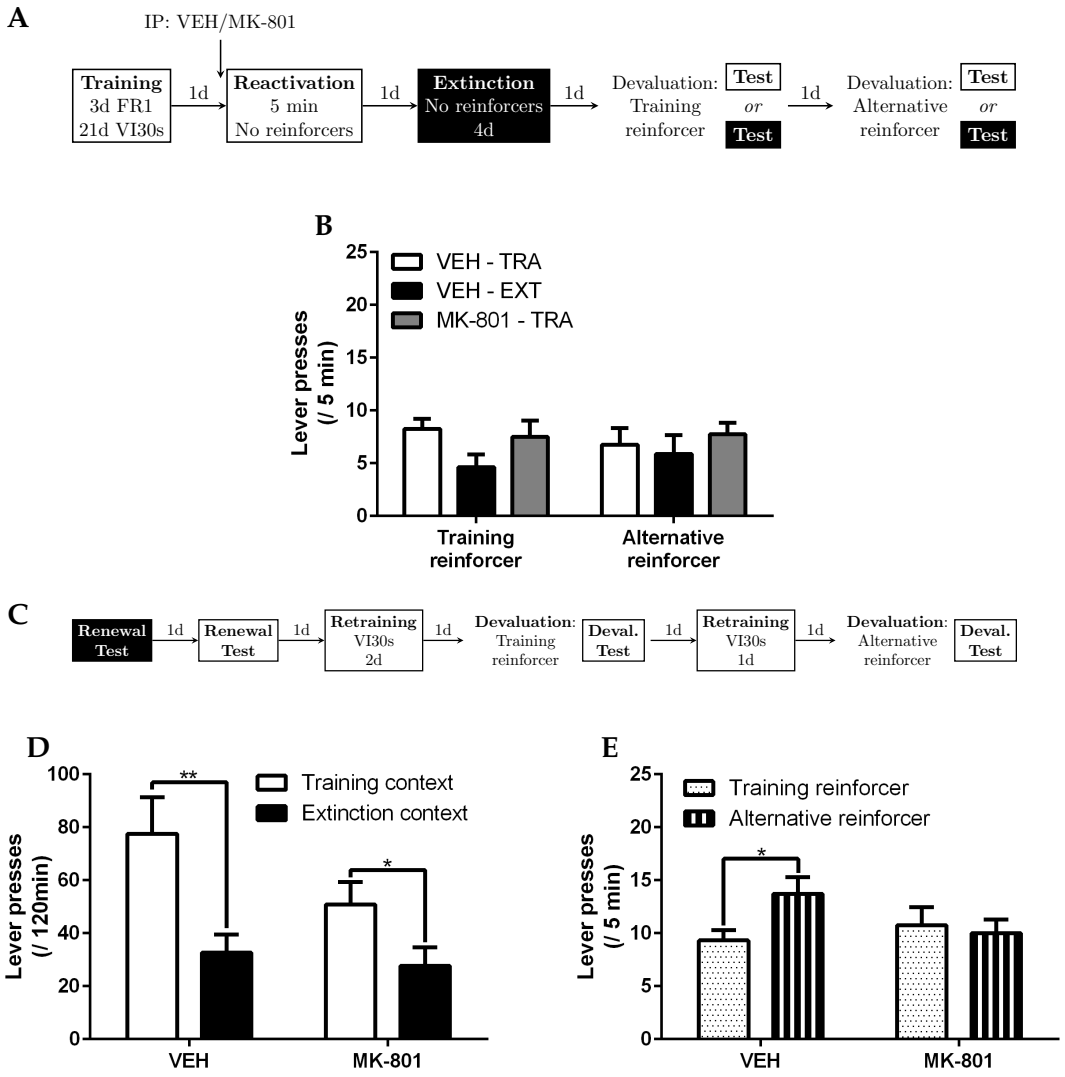


Figure 3.3: Effects of MK-801 at reactivation on renewal and expression of goal-directed behaviour. **A**: Training, reactivation and testing protocol for Experiment 2. Results of 'Test' are depicted in B. **B**: Combined testing of renewal and response to reinforcer devaluation failed to reveal any significant effects of any treatment. **C**: Additional tests conducted on animals for Experiment 2 after the initial test sessions. The results of the first two renewal tests (the first two Test boxes in C) are depicted in D whilst the results of the second devaluation (deval.) tests the second two Test boxes in C) are depicted in E. **D**: Renewed responding without prior devaluation was similar regardless of drug treatment at reactivation. **E**: Only those animals treated with vehicle at reactivation reduce lever pressing in response to reinforcer devaluation when tested after retraining. Bars represent means +SEM. N=8-16 per group. * p<.05 *, ** p<.01

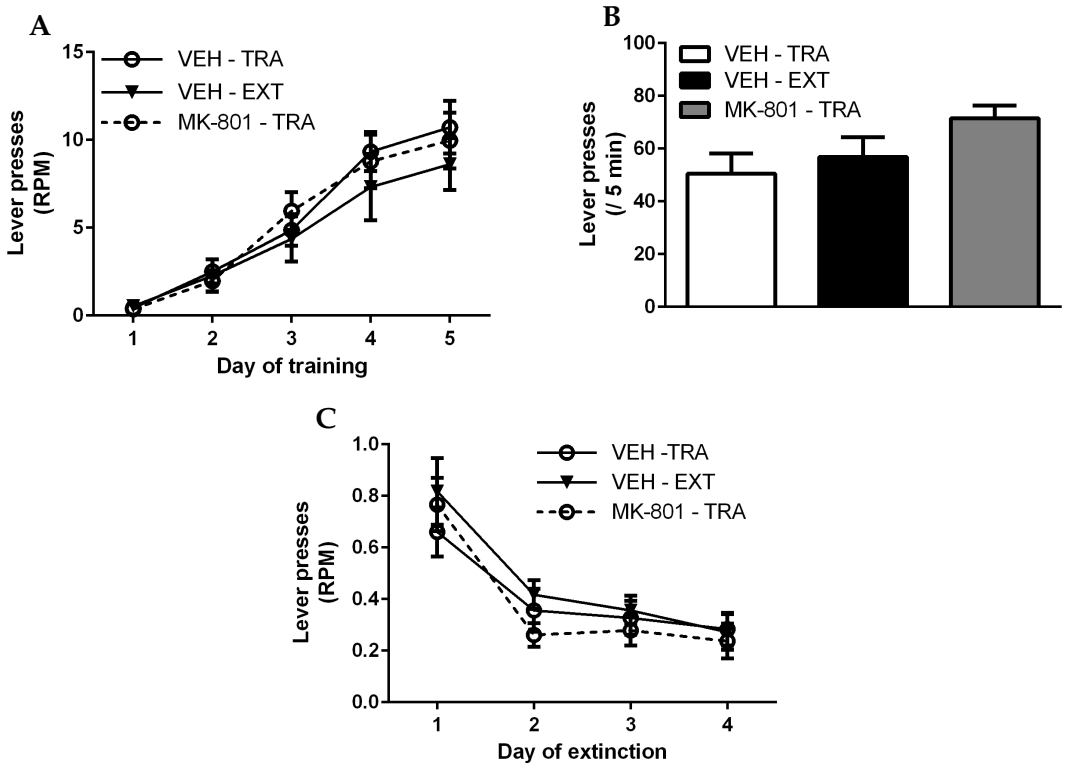


Figure 3.4: Training, extinction and reactivation data from Experiment 2. **A**: Responding during training sessions. **B**: Responses made in the reactivation session. **C**: Responses made in the extinction sessions. VEH/MK-801 refers to treatment animals had received before memory reactivation and TRA/EXT the context would subsequently be tested in. Values represent means ±/+SEM. N=8 per group.

Discussion

Summary of results

Here it is reported that responding in context-induced renewal is susceptible to devaluation after limited instrumental training. This resembles non-renewed free operant responding, which is initially governed by A-O associations and only after extended training does it become reliant upon S-R associations and insensitive to devaluation.

NMDA receptor antagonism prior to reactivation of a goal-directed memory resulted in a specific impairment in the use of the current outcome value to guide responding. This raised the possibility the retrieval session had specifically reactivated the A-O part of the memory and NMDA receptor antagonism prevented reconsolidation of this association, thus only leaving S-R associations to maintain responding.

Relationship to previous work

The ability of rodents to encode and retrieve specific relationships between actions and outcomes, whilst originally disputed (Tolman, 1933), is now relatively well-established (e.g. Adams and Dickinson, 1981; Killcross and Coutureau, 2003; Nelson and Killcross, 2006; Yin *et al.*, 2005a). Here this finding was replicated, but for responding occurring after returning animals to their training context after periods of extinction in a second environmental context.

The susceptibility of renewed responses to devaluation after limited training fulfilled the primary aim of carrying out this experiment, to develop a protocol to investigate reconsolidation of memories underlying goal-directed responding. A second experiment investigated the reconsolidation of these memories with the inclusion of a reactivation session between the training and extinction sessions. Subsequent levels of responding were apparently unaffected by NMDA receptor antagonism in the extinction sessions in the novel context. The inability of a memory reactivation session consisting of execution of an operant response to have no subsequent impact on responding is in accord with previous studies of a similar nature in animals trained to an extent such that responding is governed primarily by A-O associations (Exton-McGuinness and Lee, 2015).

Instrumental associations resulting in CIR have previously demonstrated to undergo reconsolidation (e.g. Fuchs *et al.*, 2009). With this in mind, alongside the possibility that the drug treatment at reactivation had selectively targeted the A-O memory, animals underwent a test-session that

Experiment 1 had previously demonstrated to simultaneously probe the ability to use A-O associations to guide responses and the potential influences of contextual stimuli on responding. In contrast to this initial experiment, however, there was no effect of reinforcer devaluation or context in which the test session took place, raising the possibility that the reactivation session (which was conducted in the absence of the reinforcer) resulted in similar inhibitory associations between the extinction and training contexts. The absence of a renewal or devaluation effect in vehicle treated animals precluded the assessment of whether NMDA receptor antagonism had resulted in a deficit in either of these behaviours.

In a second renewal test, conducted without prior reinforcer devaluation, vehicle treated animals responded more in the context that had been used in training. This renewed responding was observed to a similar degree in animals treated with MK-801 prior to the reactivation session, suggesting this treatment was unable to affect the reconsolidation of these responses. The previous demonstrations that memories underlying responding of this nature can undergo reconsolidation (Arguello *et al.*, 2014; Fuchs *et al.*, 2009; Wells *et al.*, 2011; Wells *et al.*, 2013) suggested that the failure of this treatment to affect responding was either the result of an inability of NMDA receptor antagonism to prevent reconsolidation, or that this process was not taking place. However, the results of a final devaluation test appeared to rule out each of these possibilities.

After the CIR sessions animals were retrained and the ability to recall A-O associations was once again assessed. Whilst animals treated with vehicle at reactivation showed intact goal-directed responding, the responses of those treated with MK-801 were apparently insensitive to devaluation of the reinforcer used in training. This raised the possibility that the retrieval session resulted in the reactivation of the A-O part of the memory and NMDA receptor antagonism prevented the reconsolidation of this trace.

This is not the first reported case whereby a reactivation session can result in a change to associative structure underlying responding. Exton-McGuinness *et al.* (2014) report that a reactivation session consisting of a shift from a predictable to unpredictable schedule of reinforcement (FR1 to variable ratio (VR)20) results in a restoration of susceptibility of responding to reinforcer devaluation with LiCl. Somewhat counter-intuitively, treatment with MK-801 prior to these sessions had no impact on this effect – responding was equally sensitive to reinforcer devaluation regardless of drug treatment at reactivation. However, there was some evidence that MK-801 administration before a reactivation session resulted in a reduced ability to detect a shift in instrumental contingencies in this study, reflective of a loss of goal-directed control (see Hammond, 1980). It appears,

therefore, that disruptions of instrumental memory reconsolidation can specifically target either the A-O (Experiment 2) or S-R (Exton-McGuinness *et al.*, 2014) part of the memory.

Disruptions of goal-directed responding can occur as a result of several manipulations such as pre-training treatment with psychostimulants such as amphetamine (Nelson and Killcross, 2006) or cocaine (Corbit *et al.*, 2014). Inactivation of the DMS prior to retrieval (Yin *et al.*, 2005a) or the PL cortex during training (Tran-Tu-Yen *et al.*, 2009) has similar effects. In contrast, inactivation of the IL cortex (Coutureau and Killcross, 2003) or DLS (Yin *et al.*, 2006) results in a restored sensitivity to devaluation in responding that would otherwise be habitual. The present data suggest that preventing memory reconsolidation of an A-O memory results in similar deficits, with animals unable to retrieve specific associations between responses and the sensory-specific properties of the reinforcer they are paired with, leaving only S-R memories to maintain responding.

Instrumental associations are not the only memories that are subserved by multiple representations in the brain. Pavlovian stimuli may influence responding either by a direct representation of the sensory-specific properties of the reinforcer paired with the CS or the ability of the general arousing properties of the stimulus to increase responding (Balleine and Killcross, 2006). The association between the CS and the reinforcer it is paired with can be probed with specific pavlovian-instrumental transfer (PIT), whereby the ability of the CS to specifically promote instrumental responding on a lever that is paired with the same reinforcer as the CS is assessed; responding that is reliant upon the BLA (Blundell *et al.*, 2001; Corbit and Balleine, 2005). In contrast, the general arousing properties of the CS can be measured with general PIT, which is reliant upon the central nucleus of the amygdala (CEN) (Corbit and Balleine, 2005; Hall *et al.*, 2001b; Holland and Gallagher, 2003). The reliance of acquisition of a new response (ANR) on the BLA[1] (Burns *et al.*, 1993) and conditioned approach upon CEN (Cardinal *et al.*, 2002; Hall *et al.*, 2001b; Parkinson *et al.*, 2000) likely indicates that these types of responding rely upon the encoding of the specific and general arousing properties of the CS, respectively.

The reconsolidation of general and specific associations between a CS and appetitive unconditioned stimuli (USs) appears to depend upon distinct neurochemical systems. Reconsolidation of the general motivational properties of the CS depends upon NMDA, but not β-adrenergic receptor activation (Lee and Everitt, 2008c; Milton *et al.*, 2012). These glutamatergic and noradrenergic systems both appear to be required for the reconsolidation of the sensory-specific properties of the

[1]The dependence of ANR on the BLA may also be parameter specific; this responding can be dichotomized into general and specific forms, like PIT (Burke *et al.*, 2008)

CS (Milton *et al.*, 2008b; Schramm *et al.*, 2016). This raises the possibility that disrupting reconsolidation of pavlovian associations with the β-noradrenergic antagonist propranolol could result in impaired specific PIT, but leave general PIT intact, an effect that would be similar to the present results. No experiments conducted to date, however, have explored this possibility with the use of a specific PIT test after reactivation.

It is important to consider some alternative explanations for the loss of goal-directed responding in animals treated with MK-801 before the memory reactivation session other than a disruption of reconsolidation of the A-O trace. It is possible that the effect of MK-801 was not due to a disruption of memory reconsolidation, but rather the result of the drug administration itself. It was only possible to detect goal-directed responding in animals following retraining after the extinction training. One possibility was that these sessions led to the formation of habitual responding exclusively in animals treated with MK-801.

Similar effects have previously been reported to occur as a result of psychostimulant administration (Corbit *et al.*, 2014; Nelson and Killcross, 2006) which, like MK-801 results in increases in striatal dopamine release (Di Chiara and Imperato, 1988; Miller and Abercrombie, 1996). These increases in dopamine (Nelson and Killcross, 2013) or possible modulation of glutamate homoeostasis (Corbit *et al.*, 2014) by MK-801 might have resulted in faster acquisition of habitual responding. However, the habit potentiating properties of psychostimulants have only been reported when they have been administered over multiple (6-7) days. Whether similar effects can be achieved with a single drug-administration has not been explored. However, given that amphetamine appears to result in much larger increases in striatal dopamine than MK-801 (Miller and Abercrombie, 1996) and these effects likely result in the accelerated habit formation in amphetamine treated animals (Nelson and Killcross, 2013), it is unlikely the present results are due to a similar effect. Inclusion of a group that received MK-801 treatment, but did not undergo a reactivation session would have helped to address this issue.

The failure of the animals treated with MK-801 to modify their response rates as a result of reinforcer devaluation may have been due to a generalisation of the decrease in drive occurring as a result of reinforcer pre-exposure. Indeed, at least numerically, it appears that the response rates of MK-801 treated animals more closely resemble those of vehicle treated group having undergone devaluation of the reinforcer used in training. Employment of a post-devaluation consumption test might have permitted the testing of this possibility. Alternatively, the use of a different reinforcer

devaluation protocol, through pairings with LiCl might prevent the possible contribution of these effects.

Implications for subsequent research

Given that the context-dependent responding was unaffected by pre-reactivation MK-801 subsequent research further investigated the effect of this treatment to result in a specific impairment in goal-directed responding without attempting to simultaneously test for CIR.

Chapter 4: The effects of NMDA receptor antagonism during retrieval of an instrumental memory

Introduction

The final experiment of Chapter 3 suggested that N-methyl-D-aspartate (NMDA) receptor antagonism prior to instrumental memory reactivation may have resulted in a selective deficit in the retrieval of an action-outcome (A-O) association. The principal aim of the experiments described in this chapter was to replicate and further explore this finding. If experiments could disrupt reconsolidation of goal-directed memories, manifested as a selective deficit in the retrieval A-O associations, future works could attempt to disrupt stimulus-response (S-R) memories in a similar fashion. Results of this nature would raise the possibility of implementing similar protocols in the treatment of substance use disorder, hypothesised to be characterised by maladaptive habits (Everitt and Robbins, 2005; Everitt and Robbins, 2016; Everitt *et al.*, 2001), to restore goal-directed control over drug-seeking.

The previous demonstration of NMDA receptor antagonism in combination with a reactivation session to result in an insensitivity of responding to reinforcer devaluation was confounded by the interposition of (re)training sessions between memory reactivation and the critical test session. This raised the possibility that the effects were the result of accelerated habit formation during these sessions, rather than deficit in memory expression resulting from a disruption of reconsolidation. Administration of drugs (in the absence of a reactivation session) prior to instrumental training has previously been shown to accelerate habit formation (Corbit *et al.*, 2014; Nelson and Killcross, 2006) and it was possible the results were caused by a similar effect. *Furthermore, the results of Chapter 3 may also have been the result of a more generalised decrease in drive in MK-801 treated animals. The use reinforcer devaluation with lithium chloride (LiCl) in this chapter aimed to rule out this possibility.*

The experiments described in this chapter therefore aimed to replicate and further characterise the impairment occurring as a result of MK-801 treatment prior to reactivation without the possible

confounds of training sessions following drug administration *and a possible generalisation account of the data obtained in the previous chapter*. Since NMDA receptor antagonism had no effect on context-induced renewal (CIR) all the training and test sessions in this chapter were conducted in the same context.

It was not possible to replicate the A-O specific retrieval deficit occurring as a result of NMDA receptor antagonism prior to memory reactivation. In order for a retrieval session to result in reconsolidation extensive evidence suggests that must be some degree of prediction error (PE) during the reactivation session. In the previous chapter this was (apparently) achieved through the absence of the expected reinforcer, in accord with previous studies (e.g. Fuchs *et al.*, 2009; Lee *et al.*, 2005b; Nader *et al.*, 2000). Whilst retrieval trials that violate learned expectancies with the unexpected reinforcer delivery (Sevenster *et al.*, 2013) or the introduction of a novel schedule of reinforcement (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014) can also result in memory reconsolidation, the omission of an expected outcome may be a favourable reactivation session in a therapeutic setting where delivery of an expected outcome may result in severe distress (in the case of post-traumatic stress disorder (PTSD)) or have legal implications (in the case of drug addiction). With these considerations taken into account all the reactivation sessions aimed to achieve sufficient PE to result in memory destabilisation with the omission of the reinforcer delivered in training.

Analysis of overall response rates in the test sessions of these experiments raised the possibility that the reactivation sessions were resulting in extinction. As discussed above, the absence of a predicted reinforcer is typically sufficient to result in memory destabilisation (e.g. Kindt *et al.*, 2009; Lee *et al.*, 2005b; Nader *et al.*, 2000). However, extensive experience of PEs results in the formation of a novel memory inhibiting the original trace (Bouton, 2004; Pavlov, 1927). Administration of amnestic agents in combination with short retrieval trials results in impairments in subsequent retrieval in comparison to vehicle treated controls. In contrast, the same treatments combined with prolonged retrieval sessions result increased memory expression in drug-treated groups, by virtue of the fact these compounds prevent the extinction learning taking place (Alfei *et al.*, 2015; Lee *et al.*, 2006b; Merlo *et al.*, 2014; Suzuki *et al.*, 2004). Retrieval trials that result in extinction do not, therefore, typically engage reconsolidation.

Recent evidence suggests that a stage exists between reconsolidation and extinction, where neither of these processes occurs, a period in which the memory is described as in 'limbo' (Merlo *et al.*,

2014). Administration of amnestic agents combined with these sessions has no impact on subsequent memory expression, nor do the trials themselves. Whilst initially characterised in discrete cued fear associations, subsequent reports have described in similar effects in contextual fear (Alfei *et al.*, 2015) and appetitive pavlovian memories (Flavell and Lee, 2013; Reichelt and Lee, 2013a). It is possible that similar processes exist for the reconsolidation of instrumental memories; retrieval trials have been previously reported for these associations that result in neither reconsolidation nor extinction (Exton-McGuinness *et al.*, 2014). Retrieval sessions that lead to reconsolidation must not result in limbo or extinction processes. The following experiments attempted to determine such parameters.

Methods

Summary

Animals were trained to lever press for a sucrose solution. After the conclusion of training they were given the opportunity to respond on this lever in the absence of reinforcer delivery, a session designed to reactivate the memory. The effects of NMDA receptor antagonism during this session were assessed with pre-reactivation MK-801 administration. In order to investigate the effects of this treatment the integrity of the A-O association was assessed. The reinforcer was devalued through pairings with LiCl, and animals' ability to recall the association between the instrumental response and the reinforcer tested (Experiments 1 & 2).

Owing to concerns that the reactivation session might be resulting in extinction, subsequent experiments used shorter reactivation sessions consisting of 25 and 10 lever presses (Experiments 3 & 4, respectively) and assessed the effect of these sessions, alongside the preceding treatment with MK-801, on subsequent responding. Animals in Experiment 5 were trained on a fixed ratio (FR)1 schedule of reinforcement throughout training and underwent a reactivation session consisting of 10 lever presses in order to investigate how the training schedule might affect the apparent rapid extinction of instrumental memories.

Procedures were conducted as in General methods except where stated.

Subjects

Subjects were 114 male Lister-Hooded rats weighing 310-375g at the start of experiments. The day before reinforcer habituation animals were food-restricted and fed 20g of rat chow at the end of each day.

Apparatus

All training took place in Med Associates operant chambers as described in General methods, with all additional contextual cues from Chapter 3 removed.

Experiments 1 & 2

Training

Training for these experiments was as in General methods, with animals receiving 3 days of FR1 and 2 days of variable interval (VI)30s training, with sessions ending after 62 minutes or when 30 reinforcers had been earned.

Reactivation

Reactivation consisted of a 7-minute session beginning with a 2-minute prequel period, as in training, followed by 5 minutes' access to the lever used during training. Responses on this lever were without consequence. At the end of the session animals were removed from operant chambers and returned to home cages. 30 minutes before these sessions animals were either administered MK-801 or its vehicle.

Devaluation and test

Animals in Experiment 1 underwent devaluation sessions involving exposure to the reinforcer in drinking cages, followed by injections of LiCl, as described in General methods. Following reinforcer devaluation in the drinking cages animals in Experiment 1 underwent a test session the next day, which was identical to reactivation session. Because this did not result in a significant devaluation effect, animals in Experiment 2 underwent further devaluation sessions within the operant chamber. This was to ensure the devaluation transferred from the drinking cages to the operant

chamber (Kosaki and Dickinson, 2010). The memory was then tested the next day, in the same way as Experiment 1.

Experiments 3 & 4

Training

Training was conducted as described in Experiments 1 & 2.

Reactivation and test

A proportion (12/24 in Experiment 3, 12/18 in Experiment 4) of the animals underwent reactivation sessions consisting of either 25 (Experiment 3) or 10 (Experiment 4) lever presses. Animals had 5 minutes to carry out the appropriate number of responses. The time limit was enforced as previous experiments demonstrated that a 5-minute reactivation did not lead to reconsolidation. 30 minutes before the reactivation session animals were injected with either MK-801 or its vehicle. Non-reactivated controls received injections and were returned to the home cage for the rest of the day.

In the test session the lever was presented for 15 minutes and responding was recorded, but was without consequence.

Experiment 5

Training

Unlike the previous experiments animals were maintained on an FR1 schedule of reinforcement for the entirety of the 5 days of training, but otherwise training parameters remained the same as in previous experiments.

Reactivation and test

Both of these sessions were conducted in Experiment 4.

Statistical analysis

Analysis was conducted as described in General methods. In experiments where there was a full factorial design (i.e. two between-subjects factors, all conditions represented) analysis was conducted with a 2*2 between-subjects analysis of variance (ANOVA). If there was not a full factorial design, responding was analysed with a one-way ANOVA with differences between groups analysed with the Šídák correction for multiple comparisons applied. Responses during training are presented as responses per minute (RPM) whilst reactivation sessions are reported as total number of lever presses. This is in order to facilitate comparison of the total number of responses made in reactivation sessions that ended after a given period of time and when sessions were limited to a certain number of responses being carried out.

Results

Experiment 1: Effects of NMDA receptor antagonism before retrieval of an action-outcome memory on responding after reinforcer devaluation

In this experiment the effects of administration of an NMDA receptor antagonist before retrieval of an instrumental memory on the subsequent expression of goal-directed memories was examined. In order to specifically test these A-O associations, the reinforcer was devalued in drinking cages with LiCl in half the rats (see Figure 4.1A).

Test results

Animals treated with MK-801 before the reactivation session made more responses at test than those treated with vehicle. This suggested that antagonism of NMDA receptors was preventing extinction, rather than reconsolidation. Responses were not decreased by reinforcer devaluation regardless of drug treatment (Figure 4.1B). This suggested that animals were habitual, although it is equally possible that the insensitivity of responding to reinforcer devaluation was the result of a failure of the LiCl pairings to transfer to the operant chamber. Treatment with MK-801 increased total number of responses made in the test session ($F_{1,20} = 6.65$, $p = .018$), but did not affect expression of the devaluation effect (Devaluation*Drug: $F_{1,20} = 1.12$, $p = .284$), with animals' responding across being insensitive to reinforcer devaluation (Devaluation: $F_{1,20} = 1.80$, $p = .195$; Figure 4.1B).

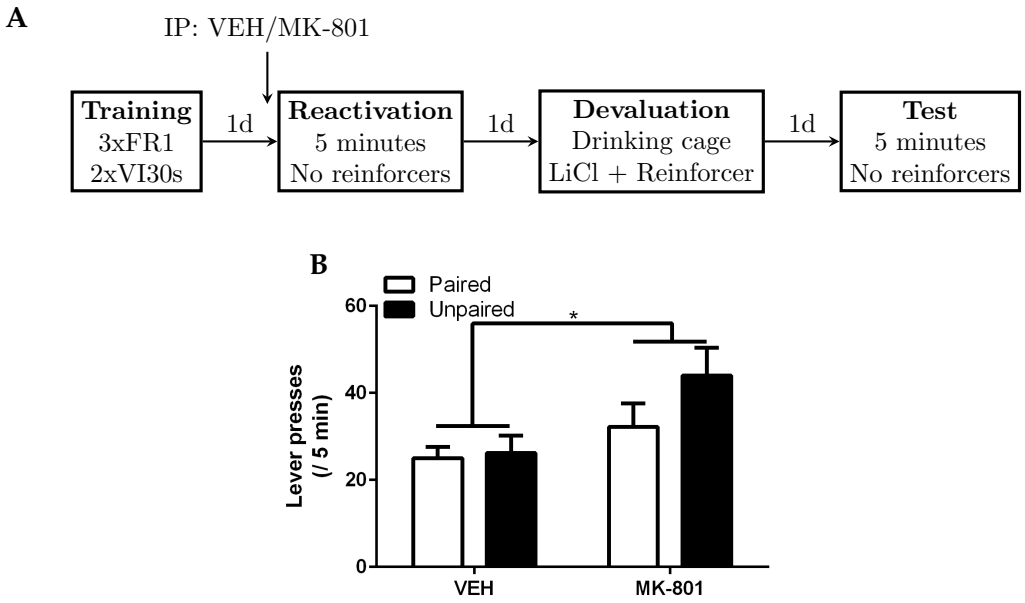


Figure 4.1: MK-801 administered before instrumental memory retrieval leads to a subsequent increase in responding for sucrose reinforcement. Lever pressing in all groups is insensitive to devaluation with pairing of the reinforcer presented in drinking cages with LiCl. **A**: Schematic of experimental procedures in Experiment 1. Only the Paired group are depicted in the diagram; the Unpaired group only received LiCl injections on days when the reinforcer was not presented. **B**: Number of lever presses made in the test session. N=6 for all groups. Bars represent means +SEM. * p<.05

Training, reactivation and devaluation

None of the effects reported in the test session were the result of pre-existing differences between the groups during training, with animals acquiring the instrumental response (Day: $F_{4,80} = 110.10$, $p < .001$) equally between treatment groups (all main effects and interactions: $F_{4,80} < 1.54$, $p > .197$; Figure 4.2A).

Rats treated with MK-801 showed higher levels of responding in the reactivation session, consistent with the known ability of this drug to result in hyperactivity (Frantz and Hartesveldt, 1999). Responses were not affected by prospective devaluation groups. This was indicated by a main effect of drug treatment ($F_{1,20} = 23.47$, $p < .001$). Prospective devaluation groups responded similarly in the reactivation session ($F_{1,20} = 0.60$, $p = .808$). The effect of MK-801 to increase responding was equal in each of these groups (Drug*Devaluation: $F_{1,20} = 0.70$, $p = .413$; Figure 4.2B).

Drug treatment prior to reactivation did not affect the acquisition of the aversion to the sucrose solution. Whilst animals in the Paired and Unpaired groups drank similar levels of the sucrose solution

on the first day ($F_{1,20} = 0.86$, $p = .364$), animals in the Paired group drank substantially and significantly less than those in the Unpaired group on the 3rd day of conditioned taste aversion (CTA) procedure ($F_{1,20} = 243.33$, $p < .001$). This was supported by a significant Day*Devaluation interaction ($F_{1,20} = 100.97$, $p < .001$). Although the overall effect of Day was similar regardless of the drug administered at reactivation (Day*Drug: $F_{1,20} = 0.75$, $p = .656$), there was a significant Day*Devaluation*Drug interaction ($F_{1,20} = 5.83$, $p = .025$). This was likely caused by an increase in consumption from the 1st to the 3rd day in vehicle treated, Unpaired animals ($t_5 = 4.20$, $p = .009$) but not MK-801 treated, Unpaired animals ($t_5 = 0.32$, $p = .759$). Importantly, there were no differences in consumption between the drug treatment groups on the final day of the CTA procedure, confirming that MK-801 treatment at retrieval did not affect acquisition of the CTA (all main effects and interactions: $F_{1,20} < 0.30$, $p > .590$; Figure 4.2C).

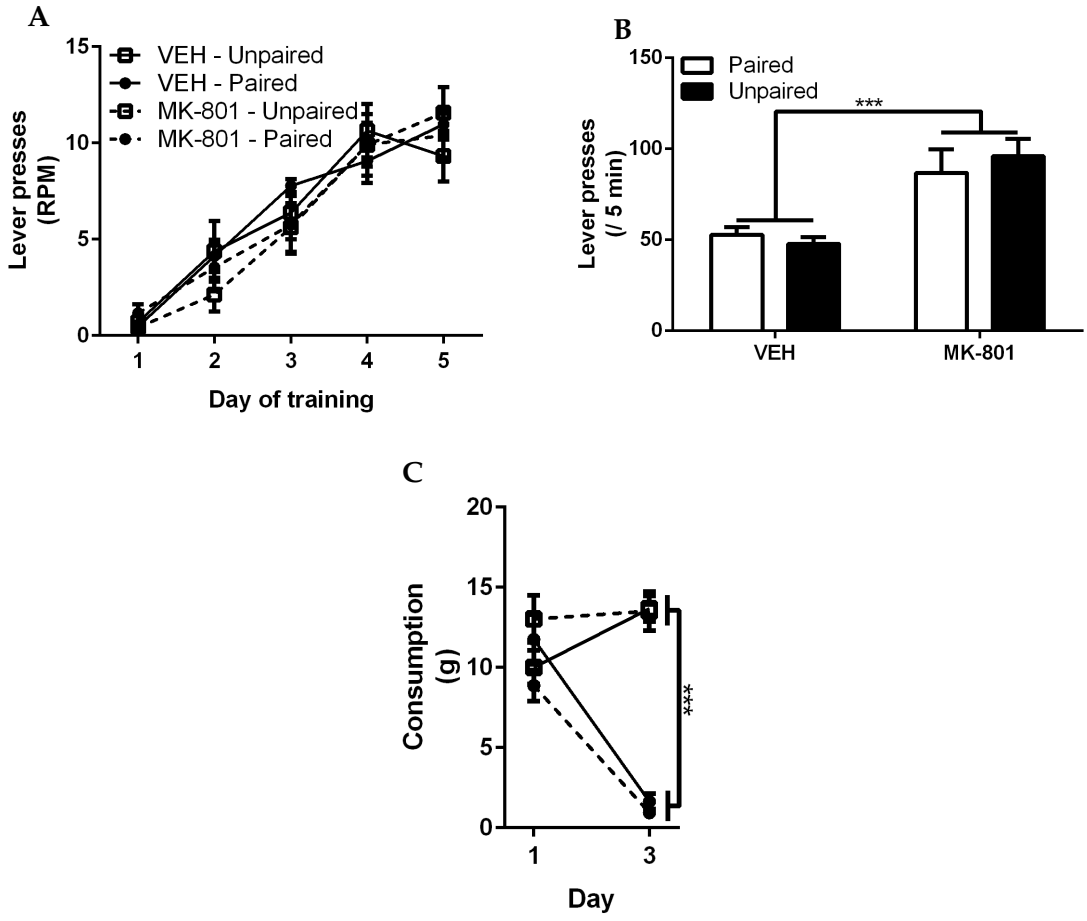


Figure 4.2: Training, reactivation and conditioned taste aversion data for Experiment 1. **A**: Rate of lever pressing in the training sessions. **B**: Number of lever presses made in the reactivation session. **C**: Consumption of sucrose in conditioned taste aversion sessions. N=6 for all groups. Data are represented as means +/±SEM. *** p<.001

Experiment 2: Effects of NMDA receptor antagonism before retrieval of an action-outcome memory on responding after additional reinforcer devaluation sessions

Since responding in Experiment 1 was insensitive to reinforcer devaluation, Experiment 2 included additional devaluation sessions in the operant chamber (see Figure 4.3A). This would test the possibility that the results of Experiment 1 were the result of a failure of the devaluation to transfer to the operant box (Kosaki and Dickinson, 2010), rather than animals being habitual. It was hoped these additional devaluation sessions would result in a robust devaluation effect, permitting investigation of whether administration of an NMDA receptor antagonist before memory reactivation prevents subsequent expression of the A-O memory.

Test results

After the additional devaluation sessions animals that had the reinforcer paired with LiCl made fewer responses in the test session, but neither the expression of this devaluation effect, nor the total number of responses, were affected by MK-801 treatment before reactivation (Figure 4.3B). This was indicated by an overall devaluation effect ($F_{1,20} = 24.04$, $p < .001$) but no Drug*Devaluation interaction ($F_{1,20} = 0.04$, $p = .849$), with both treatment groups showing a significant devaluation effect (both: $t_{10} > 2.93$, $p < .016$). Treatment with MK-801 before reactivation did not affect the number of responses made at test ($F_{1,20} = 0.39$, $p = .542$; Figure 4.3B).

Training, reactivation and devaluation

None of the results from the test session could be explained by pre-existing differences between the groups, with all groups increasing lever pressing across days of training ($F_{1.9,39.0} = 80.79$, $p < .001$) at a similar rate, regardless of the prospective drug treatment at reactivation and devaluation group (all main effects interactions: $F_{1.9,39.0} < 0.48$, $p > .619$; Figure 4.4A).

Administration of MK-801 resulted in an increase in lever pressing in the reactivation session ($F_{1,20} = 10.96$, $p = .003$). Prospective devaluation groupings did not affect the total number of responses made in the reactivation session (all main effects and interactions: $F_{1,20} < 0.47$, $p > .503$; Figure 4.4B).

Devaluation of the reinforcer with LiCl resulted in a significant decrease in its consumption on the 3rd day of the CTA training (Day*Devaluation: $F_{1,20} = 182.79$, $p < .001$; Figure 4.4C). Neither

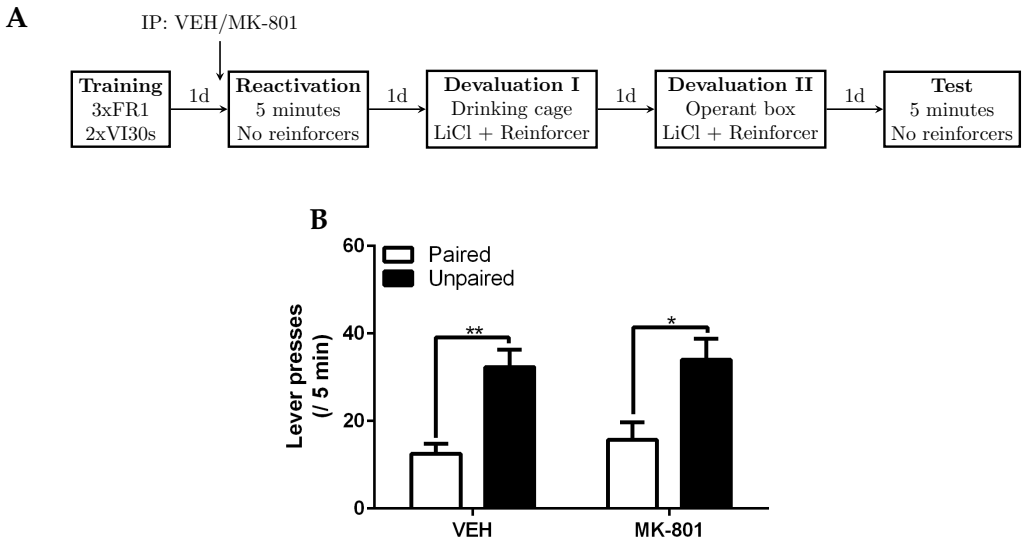


Figure 4.3: Reinforcer devaluation with LiCl in drinking cages and operant boxes resulted in expression of a devaluation effect, but this was not affected by administration of an NMDA receptor antagonist before memory reactivation. **A**: Schematic of experimental procedures in Experiment 2. **B**: Number of lever presses made in the test session. N=6 for all groups. Bars represent means +SEM. * p<.05

consumption nor acquisition of CTA was affected by MK-801 at reactivation (all main effects and interactions: $F_{1,20} < 0.64$, $p > .432$). During the additional devaluation sessions in the operant chamber Paired groups made significantly fewer nosepoke responses on both days (Figure 4.4D). There was some indication of an increase in the number of nosepokes made in the 6th day of CTA training in animals in the Unpaired group treated with MK-801 at reactivation (Day*Drug*Devaluation: $F_{1,20} = 5.76$, $p = .026$), but the difference was not significant (Day 6, MK-801–Unpaired *vs.* VEH–Unpaired: $t_{10} = 1.96$, $p = .079$; Figure 4.4D). This raised the possibility that MK-801 treatment prevented extinction of the nosepoke response during the reactivation session.

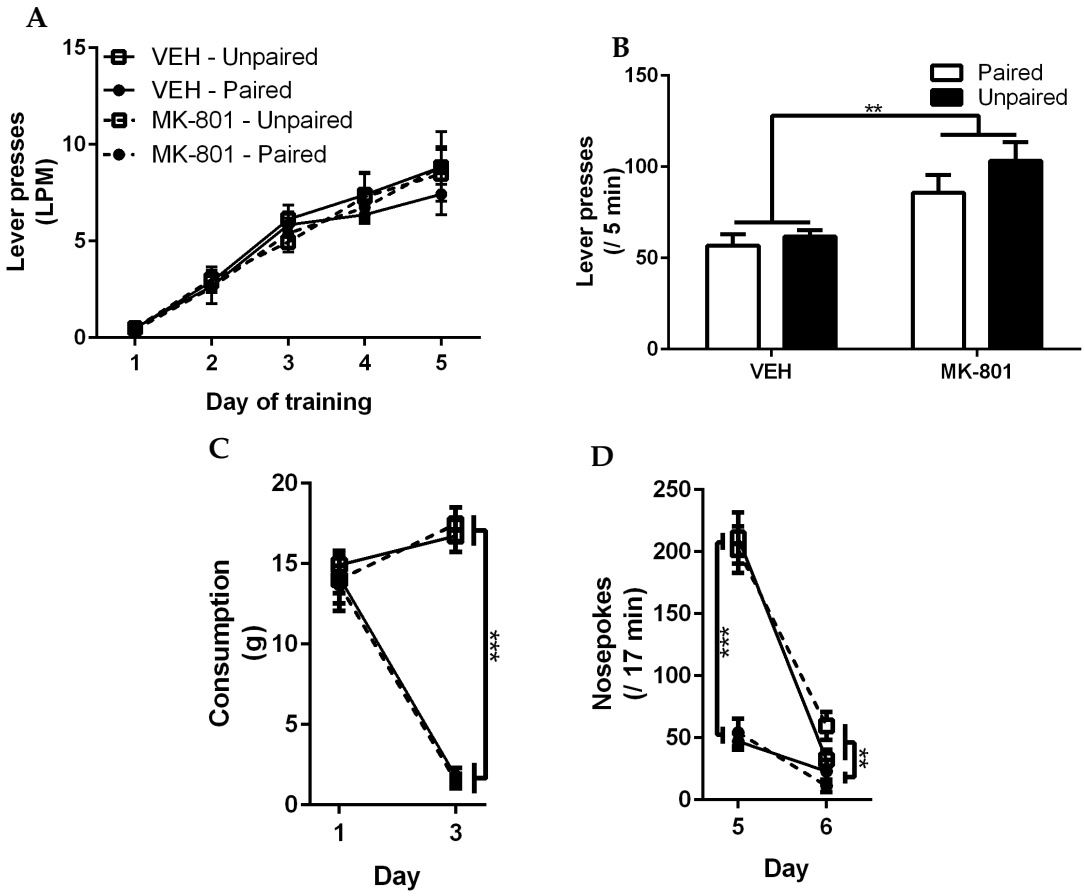


Figure 4.4: Training, reactivation and conditioned taste aversion data for Experiment 2. **A**: Rate of lever pressing in the training sessions. **B**: Number of lever presses made in the reactivation session. **C**: Consumption of sucrose in conditioned taste aversion sessions. **D**: Total nosepokes made in the 5th and 6th day of conditioned taste aversion training. Note that reinforcer was delivered on day 5, but not 6. N=6 for all groups. Data are represented as means +/±SEM. ** p<.001

Experiment 3: Effects of NMDA receptor antagonism before retrieval of an instrumental memory with 25 lever presses

Experiment 1 suggested that a 5-minute reactivation session resulted in the engagement of extinction mechanisms, rather than reconsolidation. The next experiment therefore sought to determine the parameters for a reactivation session that would not result in extinction, reducing the session length from 5 minutes to the time taken to make 25 lever presses. Because of the large number of animals required for reinforcer devaluation with LiCl, this part of the experiment was omitted whilst the optimum reactivation parameters were determined. In order to enable detection of whether the reactivation session alone was resulting in a reduction in responding (i.e. inducing extinction mechanisms) groups that did not undergo reactivation sessions were also included (see Figure 4.5A).

Test results

Analysis of the whole session suggested that treatment with MK-801, but not vehicle, led to an enhancement in responding at test when given in conjunction with a reactivation session. This rather complex pattern of results did not immediately indicate the reactivation session was resulting in extinction, since taking part in this session did not result in a decrease in responding in vehicle treated animals (Figure 4.5B). This analysis was confirmed by a significant Reactivation*Drug interaction ($F_{1,20} = 4.86$, $p = .039$), but no main effect of Reactivation ($F_{1,20} = 0.34$, $p = .568$) or Drug ($F_{1,20} = 0.00$, $p = .953$) and MK-801 treated animals that underwent a retrieval session responding more than those were also treated with this same drug but did not undergo a reactivation session (Figure 4.5B).

In order to further investigate the effects of the retrieval session and combined drug treatment on subsequent responding the first five minutes of the test session was also analysed in a similar fashion (Bin*Reactivation: $F_{2,40} = 2.46$, $p = .098$; Bin*Drug: $F_{2,40} = 0.61$, $p = .549$; Bin*Reactivation*Drug: $F_{2,40} = 2.94$, $p = .064$; see Figure 4.5B for time course). Responding in this time period is less likely to confounded by extinction occurring within the test session, which may obscure group differences (e.g. Milton *et al.*, 2008a). This revealed a slightly different pattern of results in comparison to when the whole session was analysed, with the reactivation session resulting in a decrease at responding at test in vehicle treated animals, and this decrease being blocked by administration of MK-801 (Figure 4.5C). This analysis was supported by a significant Drug*Retrieval interaction ($F_{1,20} = 6.37$, $p = .020$) but no other main effects being significant (all: $F_{1,20} < 1.73$, $p > .203$). These data suggested that the reactivation session was resulting in extinction and MK-801 was blocking this effect.

Training and reactivation

None of the results from the test session could be attributed to pre-existing differences between the groups. All groups acquired the operant response ($F_{4,80} = 60.89$, $p < .001$) at a similar rate (all interactions and main effects: $F_{3.5,70.3} = 2.49$, $p = .058$; Figure 4.6A).

There was a non-significant trend towards animals treated with MK-801 to complete the reactivation session in a shorter time than animals treated with vehicle ($t_{20} = 5.30$, $p = .089$; Figure 4.6A). All animals treated with MK-801 completed the 25 lever presses within the 5-minute time limit, whilst 2 rats from vehicle treated group failed to do so, making 14 and 23 lever presses respectively.

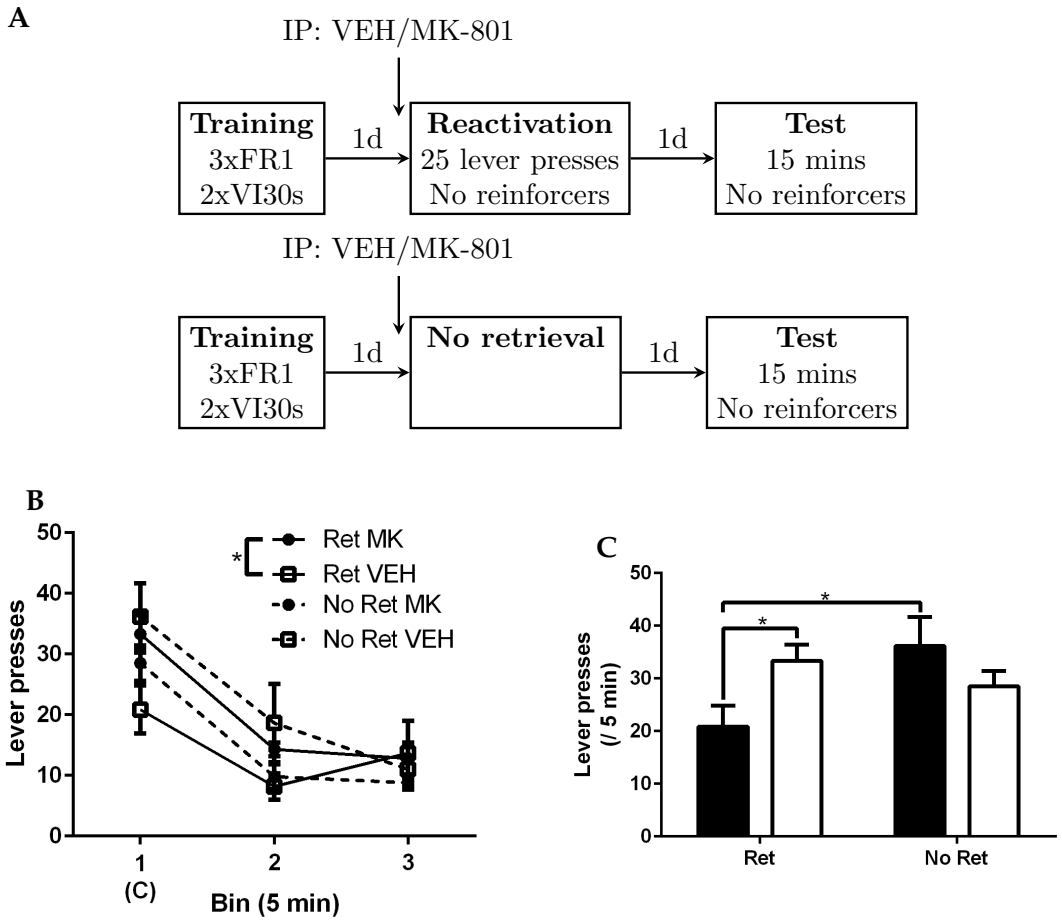


Figure 4.5: MK-801 administered before instrumental memory reactivation consisting of 25 lever presses leads to prevention of extinction, rather than reconsolidation. **A**: Schematic of experimental procedures in Experiment 3. **B**: Number of lever presses made in the test session, presented in 15-minute bins. **C**: Number of lever presses made in the first 5 minutes of the test session. N=6 for all groups. Bars represent means +SEM. * p<.05

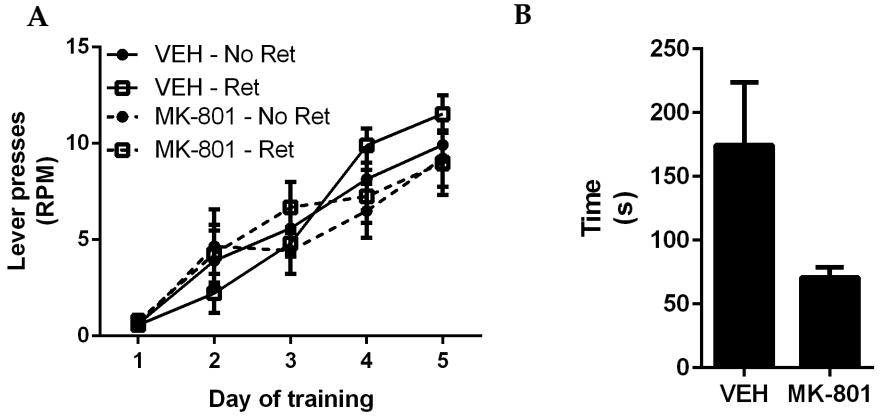


Figure 4.6: Training and reactivation data for Experiment 3. **A**: Rate of lever pressing in the training sessions. **B**: Duration of the reactivation session as a function of drug treatment. N=6 for all groups. Data are represented as means ±/+SEM.

Experiment 4: Effects of NMDA receptor antagonism before retrieval of an instrumental memory with 10 lever presses

Given that Experiment 3 demonstrated that a retrieval trial consisting of 25 non-reinforced lever presses resulted in a reduction in responding the next day, Experiment 4 further reduced the number of lever presses made in the reactivation session to 10. A proportion of animals also received treatment with MK-801 before reactivation in order to be able to detect whether reconsolidation was taking place (Figure 4.7A).

Test results

A reactivation session consisting of 10 lever presses appeared to result in a reduction at responding at test *when the whole session was analysed*. This was suggested by a non-significant trend towards Group affecting the number of responses made at the reactivation session ($F_{2,18} = 3.52$, $p = .056$). Post-hoc comparison of these groups revealed a marginal, non-significant reduction in responding in animals treated with vehicle before reactivation *vs.* their non-reactivated counterparts ($p = .056$; Figure 4.7B). Simple comparison of only vehicle treated groups with an independent samples t-test demonstrated that the reactivation session resulted in a decrease in responding at test ($t_{10} = 2.64$, $p = .025$).

As in the previous experiment, the time course of responding within the 15-minute retrieval session was also analysed, although this did not provide further clarification of the pattern of responding within the test session (see Figure 4.7B for time course). Whilst there was a trend towards a Bin*Group interaction ($F_{6,40} = 2.00$, $p = .088$), analysis of the first five minutes of this session did not reveal any group differences (or trends; $F_{2,15} = 0.73$, $p = .498$, Figure 4.7C).

The ability of the retrieval session to result in a decrease in responding suggested that extinction was taking place in the memory retrieval session. However, there was no evidence that MK-801 treatment at reactivation prevented this process. This may be the result of NMDA receptor independent learning or, more likely, the result of a modest extinction effect making an attenuation of this result difficult to detect.

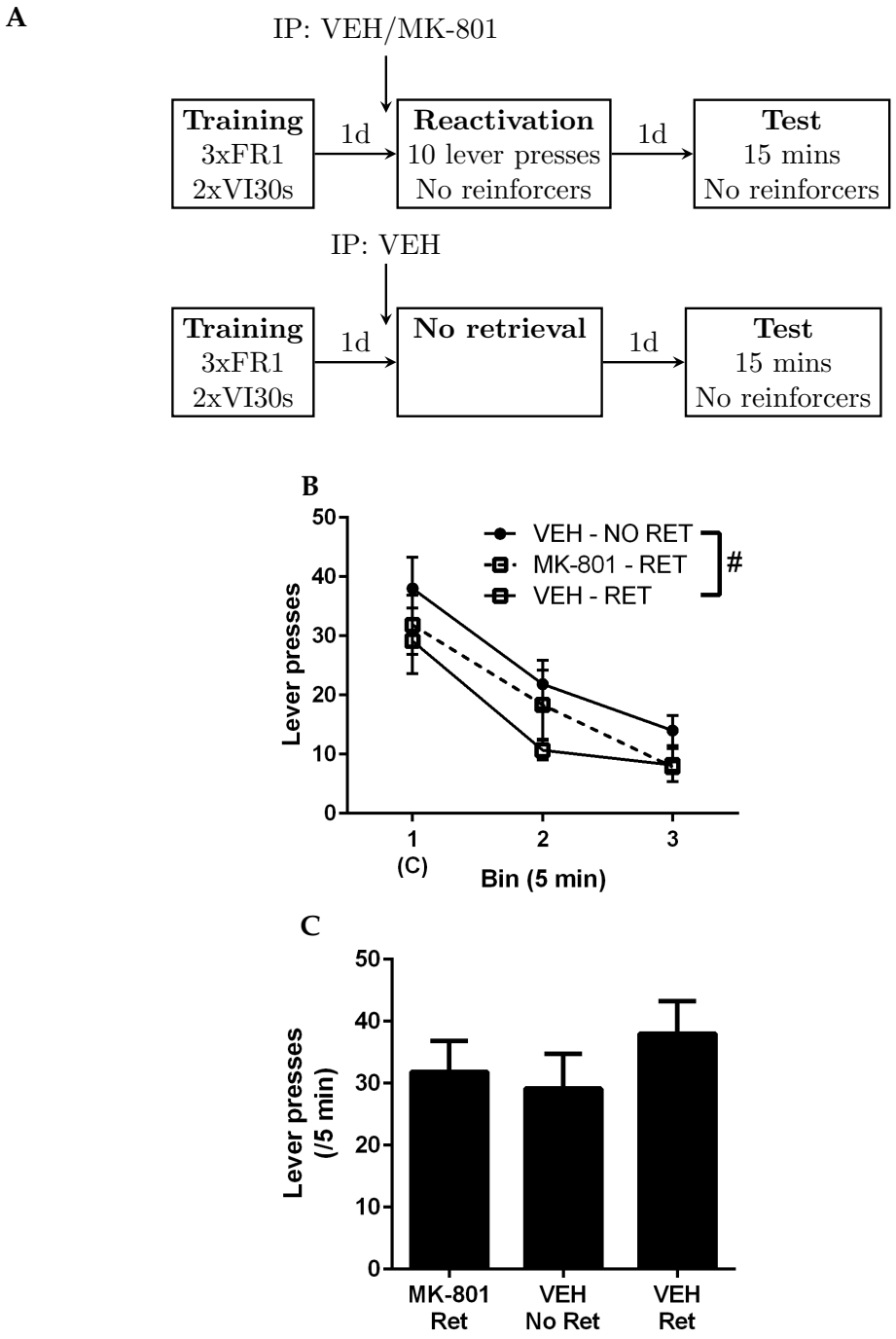


Figure 4.7: An instrumental memory reactivation session consisting of 10 lever presses appears to lead to extinction, rather than reconsolidation. **A**: Schematic of experimental procedures in Experiment 4. **B**: Number of lever presses made in the test session. **C**: Number of lever presses made in the first 5 minutes of the test session. N=6 for all groups. Bars represent means +SEM. #=p<.06

Training and reactivation

All groups acquired the operant response ($F_{4,60} = 153.91$, $p < .001$) at a similar rate (Day*Group: $F_{8,60} = 1.04$, $p = .415$) and the prospective groupings did not affect the rate of responding throughout the training sessions (Group: $F_{1,15} = 0.40$, $p = .679$; Figure 4.8A). This confirmed that the results of the test session could not be explained solely by pre-existing differences between the groups.

Prior drug treatment had no effect on the time to execute 10 lever presses ($t_{10} = 0.95$, $p = .364$; Figure 4.8B). All animals completed the 10 lever presses within the 5 minute time limit.

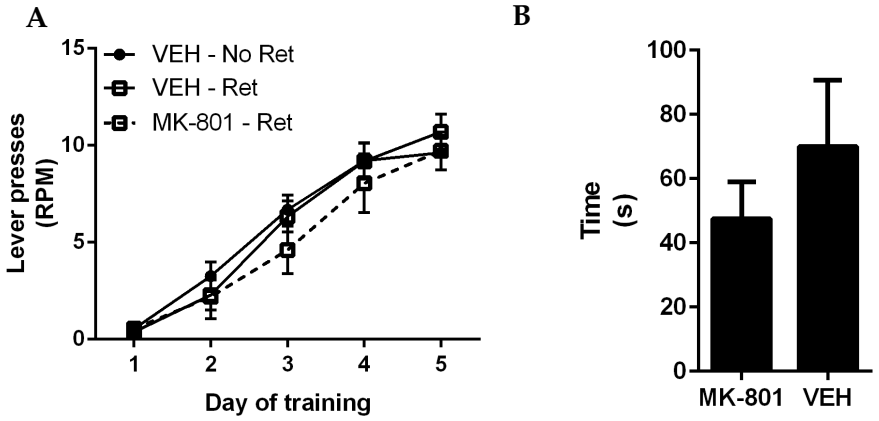


Figure 4.8: Training and reactivation data for Experiment 3. **A**: Rate of lever pressing in the training sessions. **B**: Duration of the reactivation session as a function of drug treatment. N=6 for all groups. Data are represented as means ±/+SEM.

Experiment 5: Effects of training on an FR1 schedule of reinforcement on the ability of a very short reactivation session to induce extinction

Prior experiments demonstrated that a reactivation session consisting of a very small (10-25) number of lever presses resulted in a reduction in responding in subsequent test. Whilst this suggested that extinction was taking place in these sessions, it was not expected this process would be engaged in such a short session. In Experiment 5 the possibility that the VI30s schedule of reinforcement was responsible for the decreases in responding was investigated. Inherent in this schedule is that in some cases the lever press response is not reinforced. It was possible that this led to a formation of an association whereby the operant response is not reinforced and this 'lever press-no reinforcer' association was being reactivated and strengthened in the retrieval session. It is known that similar inhibitory memories undergo reconsolidation (Eisenberg and Dudai, 2004; Rossato *et al.*, 2010) and reactivation can strengthen memories (Fukushima *et al.*, 2014; Tedesco *et al.*, 2014b).

Animals were trained on an FR1 schedule of reinforcement throughout in an attempt to reduce the likelihood of these inhibitory traces being formed and subsequently reactivated. Half of the animals underwent a reactivation session whilst the other half did not; reactivation conditions were further divided on based on whether they received MK-801 or vehicle treatment (see Figure 4.9A).

Test results

On initial observation it appeared that neither the retrieval session, nor drug treatment had any effect on the total number of responses made in the test session (all main effects and interactions: $F_{3,20} < 1.73$, $p > .202$; Figure 4.9B). When the first 5 minutes of the test session was analysed (Bin*Retrieval: $F_{1.7,33.6} = 6.88$, $p = .005$) it appeared that the retrieval session led to a decrease in responding, but this was not affected by prior drug treatment. Separate analysis of the first five minutes of the test session indicated that animals that underwent a retrieval session responded less than those that did not ($F_{1,20} = 5.81$, $p = .026$), but this was not affected by drug treatment (all main effects and interactions: $F_{1,20} < 0.65$, $p > .430$; Figure 4.9C). No other main effects or interactions were significant (all: $F_{1.7,33.6} = 2.37$, $p < .106$). Once again it appeared that the reactivation session was resulting in a decrease in responding, although in this case this was not moderated by prior treatment with MK-801.

Training and reactivation

All animals acquired the operant response ($F_{3.6,71.2} = 90.46$, $p < .001$) equally; prospective drug treatment or reactivation conditions did not affect responding at any point during training (all main effects and interactions: $F_{1,20} = 1.77$, $p = .197$; Figure 4.10A). None of the results in the test session therefore appeared to be the result of pre-existing differences between the groups.

Animals treated with MK-801 and vehicle spent a similar amount of time to reach 10 lever presses in the reactivation session ($t_{10} = 0.95$, $p = .364$; Figure 4.10B). All animals completed 10 lever presses within the time limit.

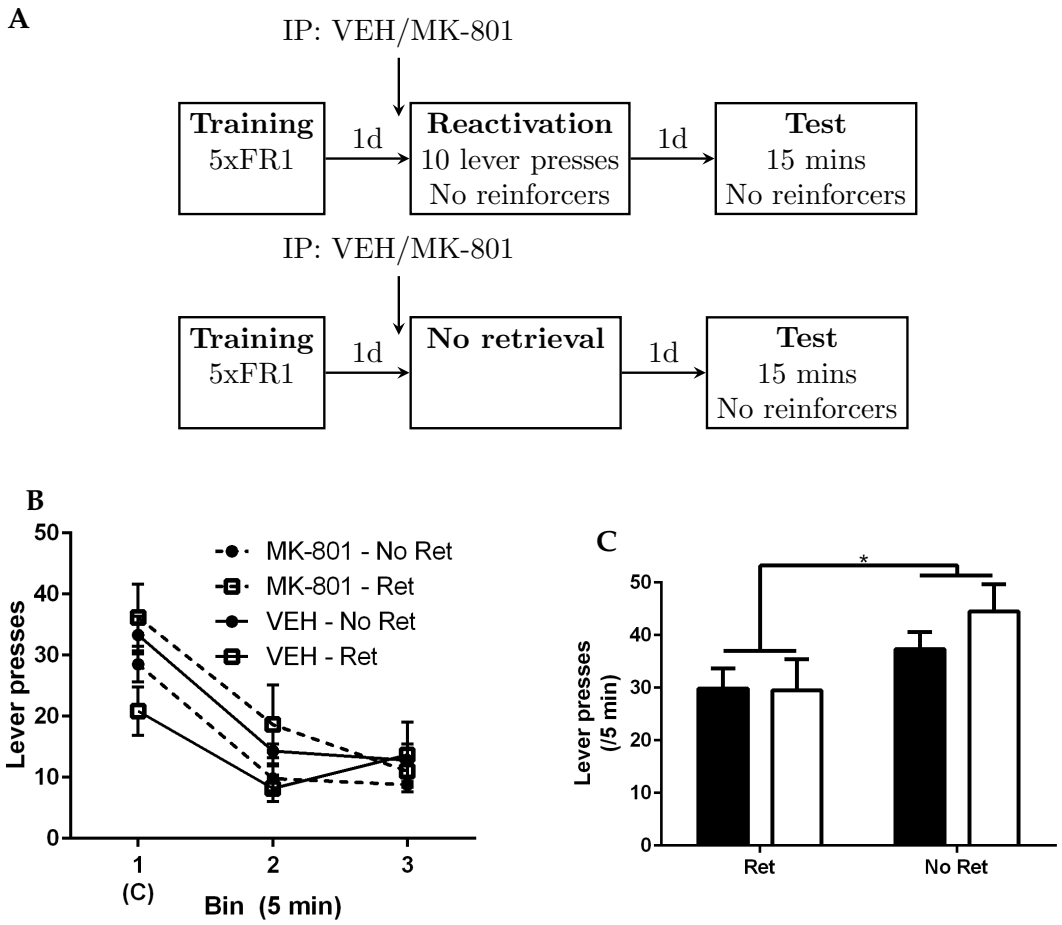


Figure 4.9: A session consisting 10 lever presses leads to a reduction in responding the next day in animals exclusively trained on an FR1 schedule of reinforcement. **A**: Schematic of experimental procedures in Experiment 5. **B**: Number of lever presses made in the test session. **C**: Number of lever presses made in the first 5 minutes of the test session. N=6 for all groups. Bars represent means +SEM. * p<.05

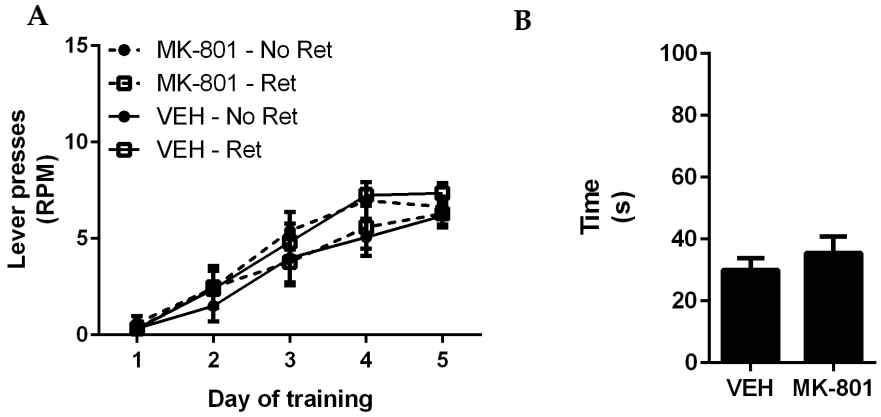


Figure 4.10: Training and reactivation data for Experiment 5. **A**: Rate of lever pressing in the training sessions. **B**: Duration of the reactivation session as a function of drug treatment. N=6 for all groups. Data are represented as means ±/+SEM.

Discussion

Summary of results

In these experiments NMDA receptor antagonism prior to retrieval of an instrumental memory did not result in an inability to retrieve A-O associations, nor did it decrease responding at test, suggesting that reconsolidation of these memories was not affected by this treatment (albeit by virtue of the fact it was not occurring in the first place). In contrast, in several experiments MK-801 treatment resulted in an increase in responding – apparently caused by the blockade of extinction – in many cases the reactivation session (in the absence of any drug treatment) resulted in a decrease in responding. This latter effect was reported despite using very short retrieval sessions.

Relationship to previous data

One difficulty in investigating instrumental memory reconsolidation is that this responding can be mediated by both action-outcome (A-O) and stimulus-response (S-R) associations. Whilst the association that governs responding is typically determined by the extent of training, evidence suggests that each of these associations are formed in parallel and where one association is lost, the other can resume control over behaviour (Balleine and O'Doherty, 2010). Reinforcer devaluation protocols, such as those used in Experiments 1 and 2, are therefore necessary in order to fully investigate a potential deficit in instrumental reconsolidation. Experiment 1 demonstrated that NMDA receptor antagonism prior to retrieval of an instrumental memory can result in an increase in responding, in comparison to vehicle treated controls. However, in this experiment it was not possible to assess whether this manipulation had affected the expression of A-O memory, owing to a failure of vehicle treated animals to reduce their responding in response to reinforcer devaluation. This was likely the result of a failure of the devaluation association to transfer to the operant chambers (Kosaki and Dickinson, 2010). The use of devaluation sessions within the operant chamber was initially avoided in order to prevent reinforcer presentation in the training context acting as a reminder of the instrumental association.

Experiment 2 included additional devaluation sessions within the operant chamber. Although the effect of MK-801 to enhance (or prevent a decrease) in responding was not replicated, both treatment groups showed intact expression of the A-O memory, as indicated by a decrease in the number of responses made in response to reinforcer devaluation with LiCl. This suggested that a 5-minute

retrieval session did not result in instrumental memory destabilisation, potentially due to the recruitment of extinction mechanisms (Experiment 1).

Given that sessions that result in extinction do not typically engage reconsolidation mechanisms (Flavell and Lee, 2013; Fuchs *et al.*, 2009; Merlo *et al.*, 2014), subsequent experiments decreased the extent of retrieval with the view of not only ensuring that extinction no longer took place, but also that reconsolidation would be engaged. Even if sessions no longer resulted in a decrease in subsequent responding this is not necessarily sufficient for reconsolidation to take place; evidence suggests a stage of 'limbo' exists between trials that result in extinction and reconsolidation (Merlo *et al.*, 2014). Despite reducing of the number of lever presses made in the reactivation sessions it still appeared that these were engaging extinction mechanisms.

The decrease in responding occurring as a result of the retrieval session may have been due to the recruitment of extinction mechanisms. In some cases MK-801 treatment prevented this effect. Extinction occurs upon repeated absence of an outcome that was previously predicted by an instrumental response and results in the formation of a new inhibitory memory (Bouton, 2004; Pavlov, 1927). Given that the sucrose reinforcer was not delivered during memory reactivation it was possible execution of the response in these sessions led to its extinction. Whilst this seems plausible for the 5-minute reactivation sessions, it seemed unlikely this was causing the reduction in responding in reactivation sessions where 10 lever presses were made. On the final day of VI30s training in Experiment 4 animals made an average of 6 responses for each reinforcer; the reactivation session only differed from training in that a single reinforcer was not delivered. Reactivation sessions that consist of a single PE result in reconsolidation, and not extinction, of pavlovian fear memories (e.g. Nader *et al.*, 2000), despite animals in these experiments only having received a single conditioned stimulus (CS)-unconditioned stimulus (US) pairing – the CS was presented with shock 50% of the time, once with the US during training, and once without during reactivation. In appetitive memories reactivation sessions consisting of CS presentation of up to 14% of those experienced in training result in reconsolidation (Lee *et al.*, 2006a). The absence of a single reinforcer in the reactivation session of Experiment 4 represented 0.7% of those experienced during training, a proportion much smaller than in previous studies where reconsolidation took place. However, it is important to acknowledge that the experiments discussed above were investigating pavlovian, rather than instrumental reconsolidation. It is possible that the dynamic between reconsolidation to extinction differs between these two memories, with instrumental associations being less resistant to the former and more amenable to the latter.

There is an emerging literature demonstrating instrumental memories do undergo reconsolidation. Notably, however, this only appears to take place when the reinforcer is delivered during memory reactivation. Weakly trained instrumental memories have been shown to reconsolidate in an NMDA receptor dependent manner following a shift from an FR1 to variable ratio (VR)5 schedule of reinforcement (Exton-McGuinness and Lee, 2015). Whilst the same manipulation was ineffective for well-trained animals, reactivations consisting of a shift from FR1 to VR20 result in reconsolidation of these memories (Exton-McGuinness *et al.*, 2014). In accord with the experiments reported here, reactivation sessions conducted in the absence of the reinforcer were reported to be without effect on subsequent memory expression, regardless of the extent of training (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014). Notably, reactivation sessions that do not differ from training do not result in instrumental memory reconsolidation (Exton-McGuinness and Lee, 2015; Hernandez and Kelley, 2004; Hernandez *et al.*, 2002), suggesting this process depends on PE in order to occur, much like several other types of memories (Alfei *et al.*, 2015; Pedreira *et al.*, 2004; Sevenster *et al.*, 2013).

The requirement for unexpected reinforcer delivery to result in the reconsolidation of instrumental associations may provide insight into the way in which these memories are updated. Theories of reinforcement learning suggest that PE signals are calculated through the use of temporal difference rules (Daw *et al.*, 2005). It is typically assumed that associations are represented by a single rule dictating the relationship between a response and reward which is updated similarly (but in opposite directions) in the face of reinforcer delivery or its absence. However, one possibility is that the likelihood of an instrumental response being executed is instead governed by competition of two rules, one for when the action is reinforced, another for when it is not. One likely candidate region in determining which of these associations governs responding is the basal ganglia, which has an integral role in carrying out instrumental actions (Balleine *et al.*, 2009), and is hypothesised to be responsible for inhibition of competing motor responses (Hikosaka, 1998; Mink, 1996). The infralimbic (IL) cortex is also important for the acquisition of inhibitory associations formed during extinction (Sierra-Mercado *et al.*, 2011). These memories have previously shown to undergo reconsolidation (Eisenberg and Dudai, 2004; Rossato *et al.*, 2010). The reinforcing and inhibitory associations are referred to as P(Response|Reinforced) and P(Response|Not-reinforced) henceforth. Retrieval sessions conducted in the absence of the reinforcer may result in the reactivation and updating of the P(Response|Not-reinforced) rule, without the need to modify the P(Response|Reinforced) rule. Given that it has previously been demonstrated that memories can become strengthened through their reconsolidation (Fukushima *et al.*, 2014; Inda *et al.*, 2011; Rohrbaugh and Riccio, 1970; Tedesco

et al., 2014b), it was possible that this reactivation led to the decrease in responding occurring during the reactivation sessions reported in this chapter. In contrast, reactivation sessions that require the updating of the P(Response|Reinforced) association, through unexpected reinforcer delivery (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014), will result of the destabilisation of this association, thus exposing it to disruption with amnestic agents. The use of VI schedule of reinforcement may have promoted the formation of these P(Response|Not-reinforced) rules.

Experiment 5 attempted to address the issue of reactivation of this hypothetical P(Response|Not-reinforced) rule with the use of FR1 schedules of reinforcement throughout the course of training, with the view that this may decrease the likelihood of the formation of these inhibitory associations. However, this revealed a similar pattern of results as when animals were trained on a VI30s schedule of reinforcement, with a short non-reinforced reactivation session continuing to result in a reduction in subsequent responding. This is in accord with published previous reports that FR1 schedules of reinforcement do not expose these memories to destabilisation with reactivation sessions conducted in the absence of the reinforcer (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014).

Implications for subsequent chapters

One of the key aims of this chapter was to characterise the reconsolidation of goal-directed instrumental memories, with the hypothesis that disruption of these associations may be manifested as a specific deficit in the retrieval of their A-O component. The intention was to then subsequently investigate reconsolidation of S-R memories with the view that it may be possible to restore goal-directed control of behaviour with disruptions of this process. Because it was not possible to disrupt reconsolidation of these associations experiments in subsequent chapters performed a similar series of investigations, targeting responses under the control of these reward paired stimuli, which, like instrumental responding in the absence of these CSs, relies upon distinct neural structures dependent on the extent of training (e.g. Murray *et al.*, 2012; Murray *et al.*, 2015).

Chapter 5: The effects of NMDA receptor antagonism during retrieval of a cocaine paired pavlovian memory

Introduction

Chapters 3 and 4 investigated the reconsolidation of instrumental memories, with the view of disrupting this process to weaken the association between an action and an outcome. Whilst these memories likely contribute to a number of psychiatric disorders, including drug addiction, these attempts were unsuccessful, apparently due to the rapid extinction occurring within the retrieval session. However, pavlovian associations also play an integral role in precipitating relapse (Everitt and Robbins, 2005; Fuchs *et al.*, 2008). The experiments in this chapter therefore aimed to characterise the reconsolidation of conditioned stimulus (CS)-drug memories with of view to disrupting their ability to maintain subsequent drug-seeking. A better understanding of the conditions in which these memories destabilise will be informative in the development of reconsolidation based treatments for drug addiction; many of the attempts to adopt this approach into the clinic have been unable to yield long-lasting decreases in craving (Das *et al.*, 2015a; Saladin *et al.*, 2013, although see Xue *et al.*, 2012).

People, places and paraphernalia paired with drug-seeking can become associated with the rewarding effects of the abused substances. These stimuli then acquire the ability to result in craving (APA, 2013). Subsequently, exposure to these CSs is a frequent cause of relapse in abstinent individuals (Fuchs *et al.*, 2008; Taylor *et al.*, 2009). Through disruptions of reconsolidation of the pavlovian associations underlying these memories their ability to promote drug-seeking can be reduced and may be a viable treatment for drug addiction.

Cues underlying drug-seeking have previously shown to undergo reconsolidation, a process that requires *zif-268* (Lee *et al.*, 2005b; Lee *et al.*, 2006a) and protein kinase A (PKA) (Sanchez *et al.*, 2010) transcription and N-methyl-D-aspartate (NMDA) receptor activation (Milton *et al.*, 2008a) within the basolateral amygdala (BLA). Systemic administration of propranolol (Milton *et al.*, 2008b),

NMDA (Milton *et al.*, 2008a) and dopamine D1 and D3 (Yan *et al.*, 2014) receptor antagonists or protein synthesis inhibitors (Dunbar and Taylor, 2016) also prevents this process.

A wide range of reactivation procedures have been reported to be effective at destabilising of memories formed during self-administration, potentially suggesting that these associations are readily destabilised, perhaps even more so than other types of memories. For example, whilst the relative novelty of contextual cues available during a memory reactivation has previously been shown to affect the ability of a cued fear memory to reconsolidate (Jarome *et al.*, 2015), memories formed during drug self-administration appear to be able to destabilise regardless of whether the reactivation session occurs in the training (e.g. Lee *et al.*, 2005a; Lee *et al.*, 2006a; Milton *et al.*, 2008a) or a novel context (e.g. Dunbar and Taylor, 2016; Sanchez *et al.*, 2010). The relationship between the number of reinforced CSs received during training affects the ability of a given retrieval session to result in reconsolidation of memories associated with food or aversive outcomes (Reichelt and Lee, 2013a; Suzuki *et al.*, 2004). However, for pavlovian associations formed during self-administration there is considerable variation in the number of CSs presentations required to result in reconsolidation; a number of CSs that is approximately 1.5 (Monsey *et al.*, 2017), 2 (Dunbar and Taylor, 2016), 6 or 14% (Lee *et al.*, 2006a) of those delivered during training have all been reported to result in memory destabilisation. Whilst contextual fear (Bustos *et al.*, 2006; Suzuki *et al.*, 2004) and avoidance (Milekic and Alberini, 2002) memories become resistant to reconsolidation with age, destabilisation of associations formed during self-administration can take place regardless of whether the memory reactivation session occurs 3 or 27 days after the conclusion of training (Lee *et al.*, 2006a). Research using sucrose reinforcers has shown that appetitive pavlovian memories only destabilise when CS-presentation is contingent upon responding, as in training (Lee and Everitt, 2008b). In contrast, both contingent and non-contingent cue delivery has shown to result in destabilisation of CS-drug memories (Lee *et al.*, 2006a; Milton *et al.*, 2008a).

From the data discussed above one might surmise that drug-paired associations are readily destabilised. Only one preclinical study has failed to prevent reconsolidation of these memories (Brown *et al.*, 2008), although it is likely these results were due to a lack of prediction error (PE) in the reactivation session (which was conducted as in training, Experiment 4) or the target memories not having the opportunity to destabilise (the cocaine paired CS was not presented, Experiment 3). Whilst not all reconsolidation effects can be attributed to publication bias (Das *et al.*, 2013), whether the paucity of studies reporting a failure of a retrieval session to result in destabilisation is due to a resistance to publish null findings or a true reflection of the ability of these memories to destabilise is unclear.

Despite the apparent ease at which CS-drug memories are destabilised in preclinical research, attempts to disrupt similar memories in addicted individuals have yielded less success. Whilst Xue *et al.* (2012) were able to reduce autonomic responses to drug-paired cues with a reconsolidation based intervention (retrieval-extinction), Das *et al.* (2015a), were unable to prevent reconsolidation with the NMDA receptor antagonist memantine, despite use of a reactivation session designed to maximise PE. Whilst Saladin *et al.* (2013) were able to reduce cue-induced craving with propranolol treatment combined with memory reactivation, this decrease was short-lived and drug treated groups showed a similar degree of craving as controls when presented with drug-paired cues a week after drug treatment. The disparity between preclinical and clinical suggests that further research is required to delineate the conditions under which CS-drug memories reconsolidate. A better understanding of the conditions in which memories formed during self-administration destabilise will help increase the efficacy of reconsolidation based treatments of drug addiction.

It was not possible to disrupt reconsolidation of CS-drug memories with non-contingent CS presentation, despite previous works suggesting that this should result in reactivation of these memories (Dunbar and Taylor, 2016; Lee *et al.*, 2006a; Monsey *et al.*, 2017; Sanchez *et al.*, 2010). Because of the evidence that sucrose-paired CSs only become destabilised with contingent CS exposure (Lee and Everitt, 2008b) experiments also used this type of reactivation, although this was also apparently unable to result in the destabilisation of the memory.

The initial experiments of this chapter used a fixed ratio (FR)1 schedule of reinforcement, where each lever press is reinforced with drug-delivery, in accord with the many experiments investigating reconsolidation of cocaine-paired CS-unconditioned stimulus (US) memories (Lee *et al.*, 2005a; Monsey *et al.*, 2017; Sanchez *et al.*, 2010). This means that the instrumental drug taking response and the cocaine-paired CS equally predict drug delivery, possibly reducing the relative ability of presentation of this CS to result in memory reactivation. In contrast, in fixed interval (FI) schedules of reinforcement, which may better reflect the prolonged periods of drug-seeking individuals must undergo to obtain drugs of abuse, reinforcement is delivered once a given period of time has passed. This means that the CS (which is only delivered alongside the drug) becomes the only predictor of cocaine. The increased ability of the CS to predict cocaine delivery may mean presentations of this stimulus are more likely to result in reconsolidation. In FR1 schedules animals can also 'titrate' the level of cocaine in their blood stream (and brain), such that a desired level of cocaine is maintained throughout the session, possibly resulting in increased associations between contextual, rather than discrete stimuli. The use of FI schedules of reinforcement prevent this titration, potentially enhancing the ability of a CS to predict cocaine delivery *vs.* that of contextual

stimuli, possibly increasing the ability of presentation of this stimulus to result in the labilisation of the CS-US memory.

The effects of MK-801 to prevent reconsolidation of a CS that was paired with cocaine during these FI training sessions were assessed with second-order schedule of reinforcement. In these schedules responses result in the delivery of reward-paired CSs; these CS presentations enhance responding during the reinforcement delays that occur during FI schedules of reinforcement (Arroyo *et al.*, 1998; Everitt and Robbins, 2000). The ability of the CS to potentiate responding was then used as a probe to assess the results of disrupting reconsolidation of a CS-US association (Lee *et al.*, 2006a).

Using reactivation and training protocols that have previously been used to result in destabilisation of a pavlovian memory formed during cocaine self-administration (Lee *et al.*, 2006a; Milton *et al.*, 2008a) it was not possible to prevent reconsolidation with the NMDA receptor antagonist MK-801. Although NMDA receptors are required for memory reconsolidation, destabilisation also depends on activation of these receptors. Intra-BLA infusion of broad-spectrum NMDA receptor antagonists prevents the destabilisation of both fear (Ben Mamou *et al.*, 2006) and conditioned-place preference (Yu *et al.*, 2016) memories. It was possible the failure to prevent reconsolidation was due to similar destabilisation-preventing effects. Studies in fear memories have shown that the GluN2B subunit is required for destabilisation (Ben Mamou *et al.*, 2006) whilst GluN2A activation is particularly important for the amnestic effects of NMDA receptor activation (Milton *et al.*, 2013). This suggests that NMDA receptor antagonists that preferentially target the GluN2A, but not GluN2B, receptor subtype will be most likely to prevent reconsolidation without affecting destabilisation. With this in mind a different NMDA receptor antagonist, CPP, was used in attempts to prevent this process, which has increased affinity of GluN2A receptors (Feng *et al.*, 2004; Feng *et al.*, 2005) and has previously has been demonstrated to prevent reconsolidation of contextual fear memories (Suzuki *et al.*, 2004).

In summary, a series of experiments attempted to disrupt reconsolidation of a pavlovian association between a visual stimulus and an intravenous cocaine infusion with NMDA receptor antagonists in combination with a memory reactivation session. A better understanding of the behavioural and neurochemical processes underlying destabilisation and reconsolidation of memories formed in self-administration in a preclinical setting will assist in developing treatments to disrupt similar memories in individuals suffering from drug addiction.

Methods

Summary

Animals were trained to respond for intravenous cocaine delivery, which was paired with presentation of a visual CS. In Experiments 1 & 2 the effects of reactivation of the resulting association with contingent and non-contingent CS presentations were compared. The effects of this treatment were assessed in a 'relapse' procedure, whereby responses led to the delivery of the CS (without prior instrumental extinction). Experiment 3 investigated the effects of MK-801 treatment prior to reactivation with non-contingent CS presentation on subsequent acquisition of a second-order scheduled of reinforcement. Experiment 4 assessed the ability of an alternative NMDA receptor antagonist, CPP, to disrupt cocaine-cue memory reconsolidation occurring as a result of non-contingent CS presentation. The effects were assessed in both the relapse procedure described above and the ability of the CS to reinstate responding after a period of instrumental extinction.

Procedures were conducted as in General methods except where stated.

Subjects

Subjects were a total of 80 Lister-Hooded rats weighing 300-425g at the time of surgery. Approximately 3 days before self-administration training began animals were food-restricted and fed 20g of rat chow at the end of each day.

Surgery

Animals underwent intravenous catherisation surgery under ketamine & xylazine anaesthesia, as described in General methods.

Training

Experiments 1,2 and 4

Animals were trained for a total of 10d to respond on a lever for intravenous infusions of cocaine (0.25mg $0.1ml^{-1}$ in sterile saline) on an FR1 schedule of reinforcement. Responses on a second lever that was present throughout training were without effect.

Experiment 3

This experiment was based on a study conducted by Lee *et al.* (2006a). Animals were initially trained on an FR1 schedule of reinforcement, as above. However, the day after animals had earned 70 CS-US pairings on this schedule they begun training on progressively leaner FI schedules of reinforcement, stabilising at FI15 (min) for 3 days (see General methods). The criterion was introduced to ensure animals in this experiment received similar number of CS-US to that of the equivalent Lee *et al.* (2006a) study. For simplicity only data for the final 11d of training are reported here (i.e. the minimum number of days to complete training) although some rats took longer because of the initial criterion.

Test and reactivation sessions

Experiment 1 & 2

Non-contingent memory reactivation consisted of 30 CS alone presentations over 30 minutes without the opportunity to respond on the levers used in training. In reactivation sessions where CS presentation was contingent upon lever pressing these were conducted exactly as in training, except that all sessions ended after 15 minutes and saline, instead of cocaine was delivered. Animals were either injected with MK-801 (Sigma-Aldrich) or its vehicle 30 minutes before these sessions, which were conducted 3 days after the conclusion of training.

Test sessions were 1 hour in duration and took place 3 days after reactivation. Active lever presses during these sessions resulted in a brief (1s) presentation of the cocaine paired stimulus but had no other consequences.

Experiment 3

Memory reactivation was with non-contingent CS presentation, as above. Animals were either injected with MK-801 or its vehicle 30 minutes before this session.

In order to investigate the ability of the CS to maintain responding in Experiment 3 animals were trained in a second-order schedule of reinforcement across the following 6 days.

Experiment 4

Memory reactivation was with non-contingent CS presentation, as above. Animals were either injected with CPP or its vehicle 60 minutes before this session.

The initial test session in Experiment 4 was as in Experiments 1 and 2. After this, animals also underwent a cue-induced reinstatement test. Animals were first given 6d of instrumental extinction, where levers were presented but responding was without consequence. The day after the conclusion of these sessions responding once more resulted in the delivery of the light CS on an FR1:S schedule. All these sessions were 2 hours in duration.

Statistical analysis

Drug and Reactivation were always treated as between-subjects factors. (Time) Bin, Day (of training), Session (e.g. FI training *vs.* second-order Day 1/6) and (presence of the) CS (in cue-induced reinstatement sessions) were coded as within-subjects factors.

Results

Experiment 1: Effects of NMDA receptor antagonism during memory reactivation with both non-contingent and contingent CS presentation

Experiment 1A: Effects of NMDA receptor antagonism with MK-801 during memory reactivation with non-contingent CS presentation

In this experiment a cocaine associated memory was reactivated through non-contingent presentation of a cocaine-paired CS under the presence of the NMDA receptor antagonist MK-801 or its vehicle. The effects of this treatment were later probed with the assessment of the ability of the reactivated CS to maintain responding (see Figure 5.1A). One animal was excluded from this experiment before testing began after its catheter became damaged during training.

NMDA receptor antagonism with MK-801 at memory reactivation had no impact on responding in the test session. Animals made similar numbers of active ($F_{1,17} = 2.69$, $p = .120$) and inactive ($F_{1,17} = 0.15$, $p = .702$) lever presses regardless of drug treatment (Figures 5.1B and 5.1D). Because within-session extinction can prevent detection of the amnestic effects of MK-801 treatment (Milton *et al.*, 2008a) the session was broken down into 15-minute time bins in order to investigate any

potential deficits in reconsolidation. This revealed a similar pattern of results, with both groups decreasing rate of active lever pressing equally within the hour session (Drug*Bin: $F_{1.9,32.1} = 1.62$, $p = .214$; Figure 5.1C). There were also no significant differences in the pattern of responding on the inactive lever (Drug*Bin: $F_{1.9,32.1} = 1.62$, $p = .214$; Figure 5.1E). MK-801 administered before memory reactivation with non-contingent CS presentation did not, therefore, appear to prevent reconsolidation, as indicated by a failure to affect responding in a test of the ability of this stimulus to maintain responding.

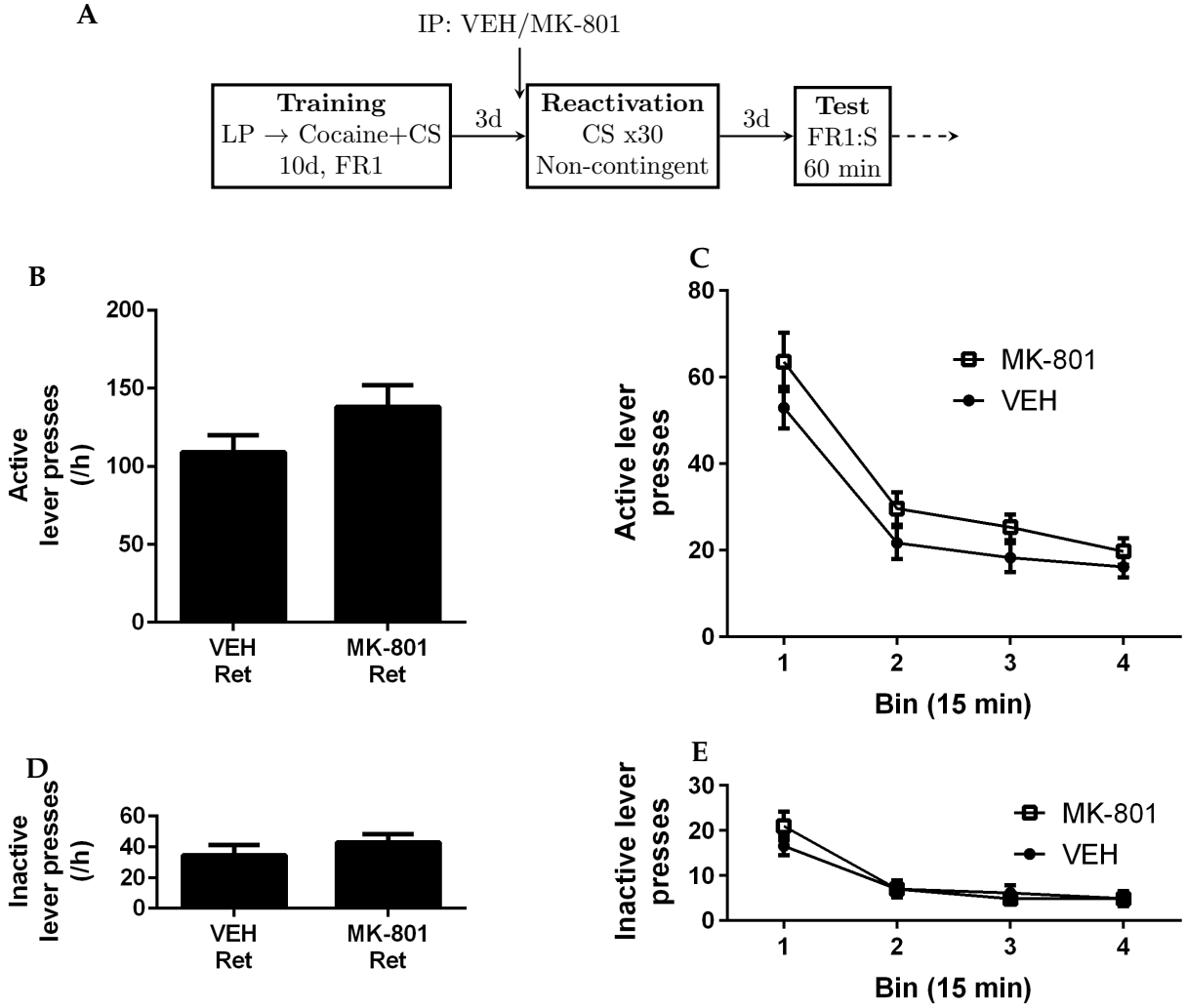


Figure 5.1: MK-801 administered before reactivation of a memory associated with cocaine with non-contingent CS exposure had no effect on subsequent cocaine seeking where responses deliver the cocaine paired CS. **A**: Schematic of experimental procedures in Experiment 1A. **B**: Total number of active lever presses made in the test session. **C**: Active lever presses from the test session, divided into 15-minute bins. **D**: Total number of inactive lever presses made in the test session. **E**: Inactive lever presses from the test session, divided into 15-minute bins. Data are represented as means +/±SEM. N=9/10 per group.

Experiment 1B: NMDA receptor antagonism during a second memory reactivation with contingent CS presentation

After the first test animals underwent a second reactivation session, with CS presentation now being contingent upon lever pressing (see Figure 5.2A). This type of reactivation has previously shown to increase the likelihood of an appetitive memory being destabilised (Lee and Everitt, 2008b). Further reactivation sessions have also been shown to make memories more amenable to reconsolidation blockade (Robinson and Franklin, 2010). Drug administration and treatment groups were exactly as in Experiment 1A. One animal from the MK-801 group was excluded from this part of experiment after its catheter became blocked during retraining.

Whilst overall response rates in the second test were not significantly affected by drug treatment at reactivation, further inspection of responding within the session, broken down into time bins, revealed that MK-801 treatment appeared to increase, or prevent a decrease, in responding. Analysis of responding across the whole session revealed a trend towards MK-801 treated animals responding more on the active ($F_{1,16} = 3.94$, $p = .064$; Figure 5.2B) but not inactive ($F_{1,16} = 0.98$, $p = .337$; Figure 5.2D) lever than those treated with vehicle. Although analysis of the session in 15-minute time bins did not yield a significant Bin*Drug interaction ($F_{1.9,31.0} = 2.64$, $p = .089$) analysis of only the first 15 minutes of the session did reveal a significant increase in the number of active lever presses made in the MK-801 group in comparison to animals treated with vehicle (Figure 5.2C). Treatment with MK-801 before the second reactivation had no effect on the pattern of inactive responses made in the test session (Bin*Drug: $F_{1.6,25.3} = 0.26$, $p = .725$; Figure 5.2E).

Experiment 1: Training, retraining and reactivation

None of the results of the test sessions could be explained by pre-existing differences in the treatment groups. All prospective groups learned to respond on the active lever in a similar fashion (Day*Drug: $F_{2.8,47.9} = 0.58$, $p = .623$; Figure 5.3A) with prospective drug treatment not affecting the response rate on the active ($F_{1,17} = 0.20$, $p = .889$) or inactive ($F_{1,17} = 0.45$, $p = .834$) lever during training. Animals in both groups showed a similar pattern of inactive lever pressing during training (Day*Drug: $F_{3.4,57.5} = 1.58$, $p = .126$; Figure 5.3B). There were also no differences in the number of CS presentations earned during the training sessions between the prospective groups ($F_{1,17} = 0.43$, $p = .521$; Figure 5.3C).

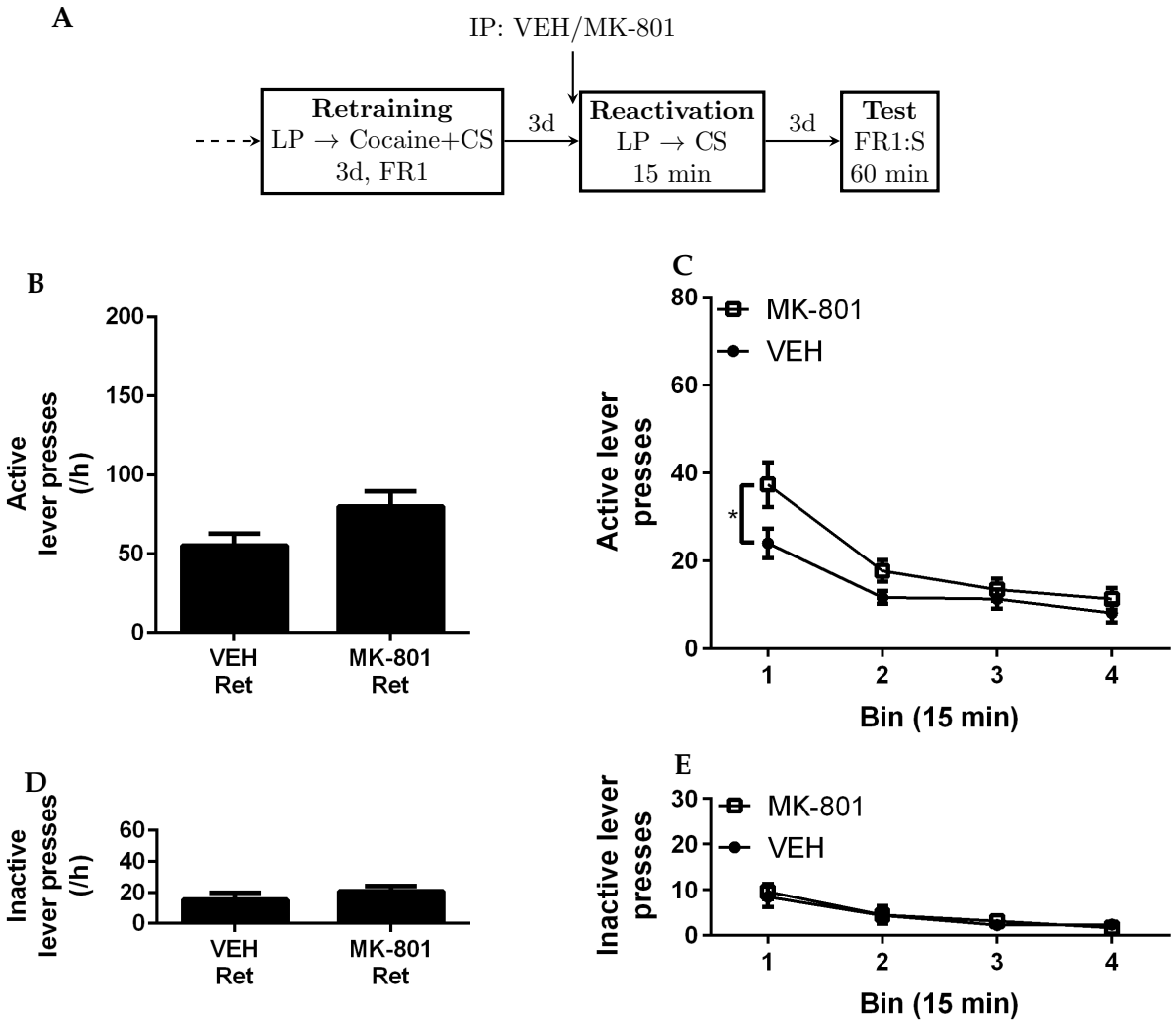


Figure 5.2: MK-801 administered before a second reactivation of a memory associated with cocaine with contingent CS exposure resulted in increased responding in subsequent cocaine seeking where responses deliver the cocaine paired CS. **A**: Schematic of experimental procedures in Experiment 1B. **B**: Total number of active lever presses made in the second test session. **C**: Active lever presses from the second test session, divided into 15-minute bins. **D**: Total number of inactive lever presses made in the second test session. **E**: Inactive lever presses from the second test session, divided into 15-minute bins. Data are represented as means +/±SEM. N=9 per group * p<.05

Drug treatment at reactivation had no effect on the reacquisition of active lever pressing after the first test (Drug*Day: $F_{1.1,17.1} = 1.69$, $p = .212$), with no main effect of Drug on the number of active ($F_{1,16} = 0.21$, $p = .656$; Figure 5.3A) or inactive lever presses ($F_{1,16} = 0.66$, $p = .428$; Figure 5.3B). There were also no differences in the total number of CSs earned in these sessions between drug groups ($F_{1,16} = 1.36$, $p = .261$; Figure 5.3C).

In the second reactivation session pre-treatment with MK-801 resulted in an increase in active ($F_{1,16} = 12.39$, $p = .003$; Figure 5.3D) but not inactive lever pressing ($F_{1,16} = 2.08$, $p = .169$; Figure

5.3E). Thus, animals treated with MK-801 received more presentations of the CS in the reactivation session than those treated with vehicle (VEH: 17.5 ± 0.94 *vs.* MK-801: 27.3 ± 2.67; $F_{1,16} = 11.91$, $p = .003$).

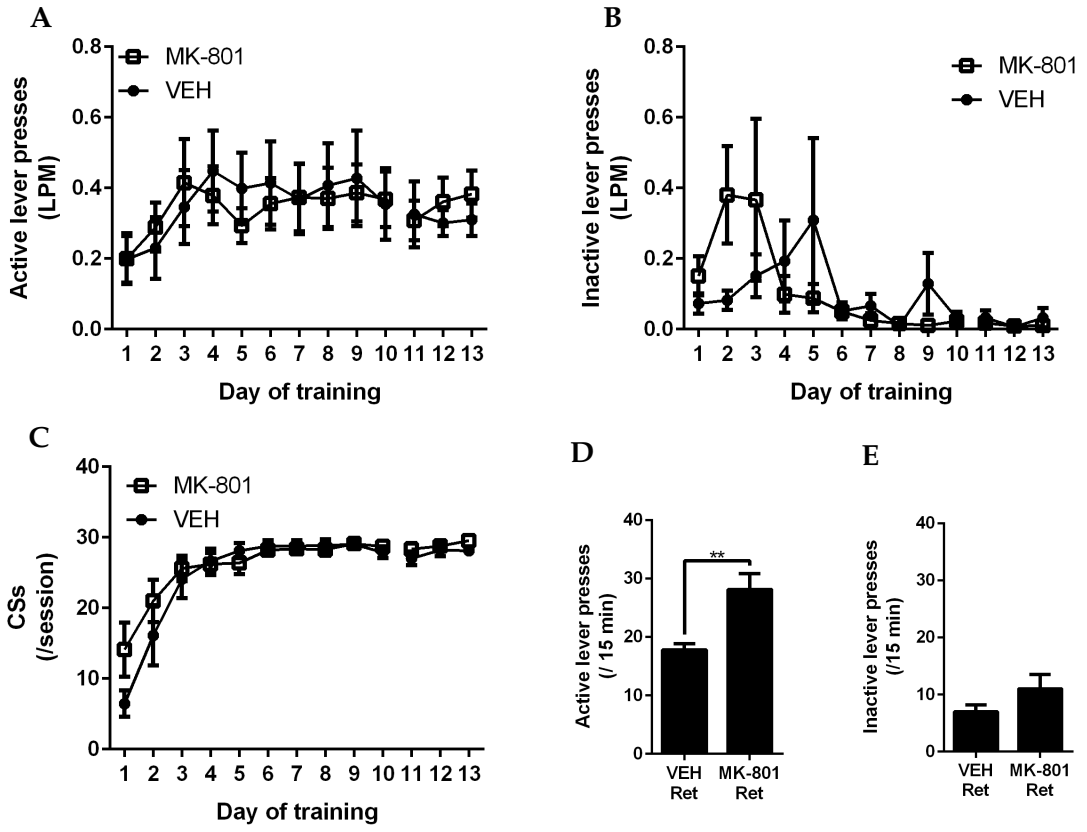


Figure 5.3: Training and reactivation data for Experiment 1. **A**: Rate of active lever pressing during training sessions. **B**: Rate of inactive lever pressing during training sessions. **C**: Conditioned stimuli earned in the training sessions. **D**: Rate of active lever pressing in the second reactivation session. **E**: Rate of inactive lever pressing in the second reactivation session. Data are represented as means +/±SEM. N=9/10 per group. ** p<.01

Experiment 2: Effects of NMDA receptor antagonism with MK-801 during memory reactivation with contingent CS presentation

Experiment 2 further investigated the effects of NMDA receptor antagonism before memory reactivation with behaviourally contingent CS presentation on the subsequent response potentiating properties of the CS in a later test (see Figure 5.4A), with the view that this type of retrieval session might be more likely to result in memory destabilisation (Lee and Everitt, 2008b).

Test results

NMDA receptor antagonism prior to the memory reactivation session had no effect on subsequent responding at test, suggesting the value of the CS was not affected by this treatment. This was indicated by the number of active lever presses made in the test session being similar between the two drug groups ($F_{1,18} = 0.58$, $p = .456$; Figure 5.4B). The number of inactive lever presses made in the test session was not affected by MK-801 treatment at reactivation ($F_{1,18} = 0.46$, $p = .508$; Figure 5.4D). There was a similar pattern of results when the session was divided into 15-minute bins, with both drug treatment groups showing similar patterns of active (Bin*Drug: $F_{1.7,31.0} = 0.19$, $p = .793$; Figure 5.4C) and inactive lever pressing (Bin*Drug: $F_{2.0,36.0} = 1.90$, $p = .164$; Figure 5.4E) in the session. This pattern of results suggested that contingent CS presentation was not effective at triggering reactivation of the CS-cocaine memory formed during self-administration.

Training and reactivation

The results described above were not confounded by pre-existing differences between prospective treatment groups. All groups responded on the active lever at similar rates during training (Drug: $F_{1,18} = 0.41$, $p = .530$; Day*Drug: $F_{2.4,43.3} = 1.49$, $p = .236$; Figure 5.5A). There were also no differences in inactive lever pressing throughout training (Drug: $F_{1,18} = 0.97$, $p = .339$; Day*Drug: $F_{2.5,45.5} = 0.56$, $p = .614$; Figure 5.5B). Both groups received similar number of CS-US associations during training (Drug: $F_{1,18} = 0.11$, $p = .750$; Figure 5.5C).

MK-801 had no effect on the total number of active lever presses made in the reactivation session ($F_{1,18} = 0.08$, $p = .779$; Figure 5.5D). Drug treatment did, however, affect the total number of inactive lever presses made in the session ($F_{1,18} = 17.34$, $p < .001$), with MK-801 treatment resulting in an unexpected *decrease* in inactive lever pressing (Figure 5.5E). Both groups received an equal number of CS presentations in the reactivation session (VEH: 24.5 ± 2.36 *vs.* MK-801: 24.1 ± 2.36; $F_{1,18} = 0.01$, $p = .906$).

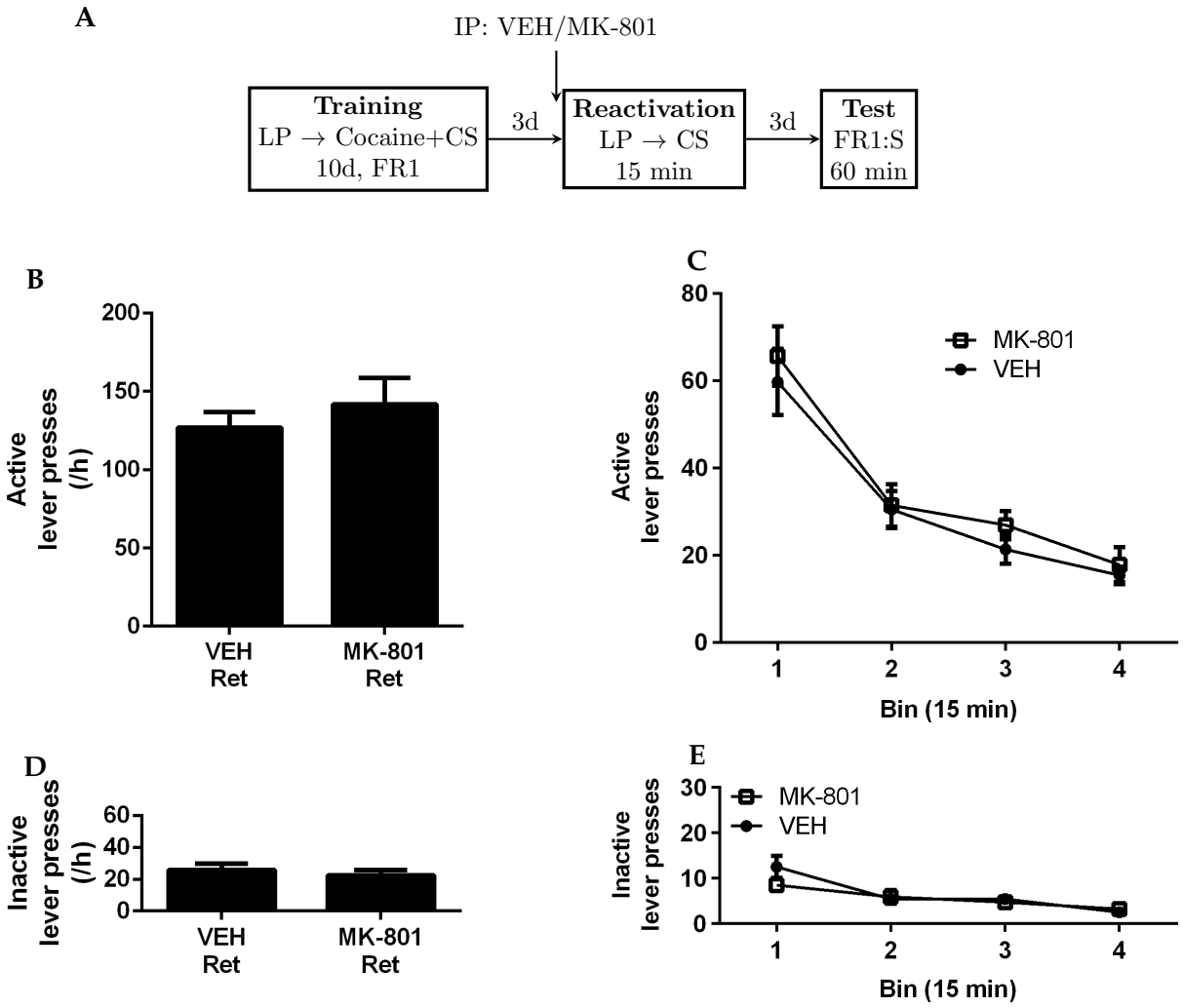


Figure 5.4: MK-801 administered before reactivation of a memory associated with cocaine using CS exposure contingent on lever pressing had no effect on subsequent on the ability of this stimulus to maintain responding. **A**: Schematic of experimental procedures in Experiment 2. **B**: Total number of active lever presses made in the test session. **C**: Active lever presses from the test session, divided into 15-minute bins. **D**: Total number of inactive lever presses made in the test session. **E**: Inactive lever presses from the test session, divided into 15-minute bins. Data are represented as means +/±SEM. N=10 per group.

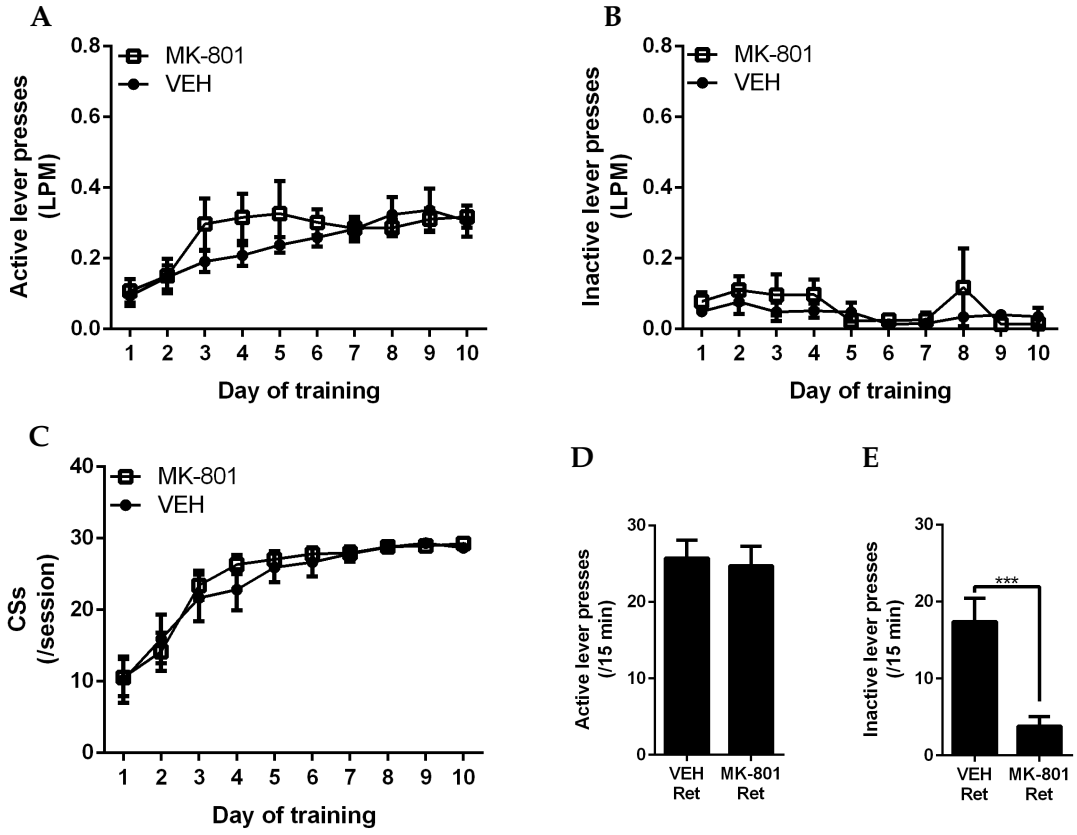


Figure 5.5: Training and reactivation data for Experiment 2. **A**: Rate of active lever pressing during training sessions. **B**: Rate of inactive lever pressing during training sessions. **C**: Conditioned stimuli earned in the training sessions. **D**: Total number of responses on the active lever in the reactivation session. **E**: Total number of responses on the inactive lever in the reactivation session. Data are represented as means +/±SEM. N=10 per group. *** p<.001

Experiment 3: Effects of NMDA receptor antagonism with MK-801 during memory reactivation with non-contingent CS presentation on subsequent acquisition of a second-order schedule of reinforcement

After training on fixed interval schedules of reinforcement a proportion of animals underwent a reactivation session, with each half of the reactivated and non-reactivated groups receiving MK-801 or its vehicle. Over the following 6 days the ability of this stimulus to maintain responding in a second-order schedule of reinforcement was assessed (see Figure 5.6A).

Effects on total number of responses made in first and subsequent intervals

In day one of second-order training all animals made more lever presses in comparison to baseline performance (without contingent CS presentation) during the first interval. Importantly, this effect was of a similar magnitude in the different drug and reactivation groups. This was indicated by animals responding more in the first interval of the first session of second-order training ($F_{1,20} = 18.16$, $p < .001$). This increase was uniform between groups, regardless of reactivation and drug treatment (Drug*Reactivation*Day: $F_{1,20} = 0.17$, $p = .897$; Figure 5.6B). The same pattern was revealed in the other intervals (Day: $F_{1,20} = 15.10$, $p = .001$; Drug*Reactivation*Day: $F_{1,20} = 2.63$, $p = .935$; Figure 5.6C). No other interaction or main effect was significant for either the first or subsequent intervals ($F_{1,20} < 1.62$, $p > .220$).

On the sixth day of second-order training animals continued to show increased lever pressing in the first interval in comparison to baseline performance. However, those treated with MK-801 responded more than their vehicle treated counterparts and animals that did not undergo a reactivation session. This was supported by an overall effect of Day ($F_{1,20} = 15.43$, $p = .001$) and this effect differing between treatment groups (Drug*Day: $F_{1,20} = 6.69$, $p = .016$). Although there was not a significant Drug*Reactivation*Day interaction: ($F_{1,20} = 1.20$, $p = .287$), given that MK-801 does not typically affect cue-maintained cocaine seeking when given in the absence of a reactivation session (Milton *et al.*, 2008a) the effects of Drug were analysed separately in the reactivated and non-reactivated groups.

Whilst drug treatment affected acquisition of a second-order schedule of reinforcement in animals that underwent a reactivation session (Day*Drug: $F_{1,14} = 10.40$, $p = .006$) this was not true of animals that did not (Day*Drug: $F_{1,6} = 0.86$, $p = .390$). Further analysis revealed that at this later stage of second-order training only animals treated with MK-801 before a reactivation session increased

their responding in comparison to baseline, suggestive of a memory enhancing effect of MK-801. Animals treated with MK-801 at retrieval responded at a greater level than animals that did not undergo a memory reactivation session (Figure 5.6D).

These effects were not seen in the other intervals; animals increased their lever pressing in comparison to baseline ($F_{1,20} = 41.45$, $p < .001$), but this increase was not moderated by drug treatment and/or whether animals underwent a memory retrieval session (all interactions: $F_{1,20} < 1.93$, $p > .179$). Furthermore, all groups appeared to increase their lever pressing in comparison to baseline, although the difference did not always reach statistical significance (Figure 5.6E).

The increases in responding occurring as a result of MK-801 treatment were not caused by a general increase in locomotor activity. Although introduction of the second-order schedule of reinforcement did result in a small increase in the number of inactive lever presses on the first day ($F_{1,20} = 8.02$, $p = .010$) this did not interact with any other factor (all: $F_{1,20} < 1.25$, $p > .276$; see Table 5.1). There were no differences between baseline and the sixth day of second-order in inactive lever presses made in the first interval ($F_{1,20} = 0.98$, $p = .335$) and this was not affected by reactivation and/or drug treatment (all interactions: $F_{1,20} < 1.71$, $p > .204$; see Table 5.1).

Interval Group	Baseline	Test	Test II
1			
VEH No Ret	7.8 ± 2.54	13.8 ± 5.63	9.5 ± 2.53
MK-801 No Ret	5.3 ± 0.32	10.0 ± 1.08	8.8 ± 1.49
VEH Ret	7.6 ± 1.12	12.4 ± 1.05	9.9 ± 2.19
MK-801 Ret	10.4 ± 2.46	10.4 ± 2.72	7.9 ± 1.98
2-5			
VEH No Ret	4.6 ± 1.70	7.9 ± 2.56	7.6 ± 3.14
MK-801 No Ret	12.2 ± 2.23	13.8 ± 3.03	14.7 ± 4.62
VEH Ret	10.3 ± 2.79	13.7 ± 2.83	15.6 ± 2.71
MK-801 Ret	9.6 ± 1.96	11.5 ± 3.61	10.9 ± 2.72

Table 5.1: Inactive lever presses made in 3 stages of second-order training. Baseline refers to responding before introduction of the second-order schedule of reinforcement. Days 1 and 6 refer to the number of days of training in the second-order schedule. 2-5 refers to the average number responses made in these intervals. Values represent mean values $\pm$ SEM to 2 decimal places.

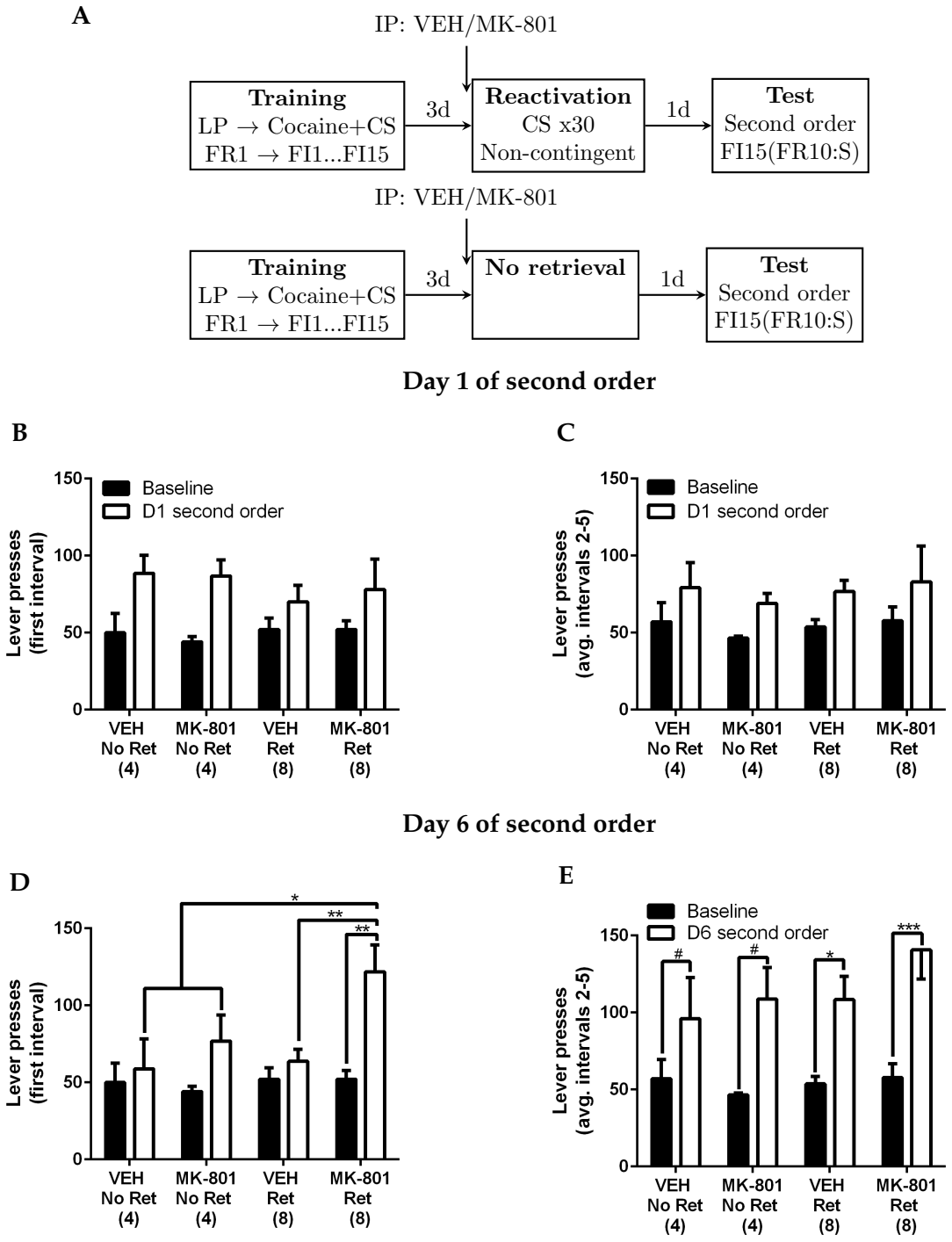


Figure 5.6: MK-801 administered before a reactivation session of consisting of non-contingent CS exposure resulted in an enhancement in subsequent responding in a second-order schedule of reinforcement. **A**: Schematic of experimental procedures in Experiment 3. **B**: Responding on the active lever during the first interval on the first day of second-order. Significance not plotted. **C**: Responding on the active lever during the mean of the 2nd-5th intervals on the first day of second-order. Significance not plotted. **D**: Responding on the active lever during the first interval on the sixth day of second-order. **E**: Responding on the active lever during the mean of the 2nd-5th intervals on the sixth day of second-order. Data are represented as means +/- SEM. The numbers beneath of the bars represent the N of each group. # p<.08, * p<.05, ** p<.01, *** p<.001

Effects on latency to reach 10th active lever press

In order to investigate whether the increases in responding seen from baseline to second-order conditioning were the result of introduction of response contingent CSs or non-specific effects (e.g. abstinence, additional training sessions), the time taken to make 10 active lever presses was analysed in a similar fashion to responding in the first interval. This can be used to provide a measure of drug seeking unaffected by CS presentation, since it is only on the 10th lever press that the cocaine-paired stimulus is presented. Analysis of these data suggested that none of the differences in responding reported in the test sessions could be explained by factors unrelated to CS presentation (Figure 5.7), details of which are reported below.

Unlike the total number of responses, introduction of the second-order schedule of reinforcement had no detectable effect on the latency to make the tenth lever press on the first day of second-order ($F_{1,20} = 0.26$, $p = .616$; Figure 5.7A). Although no interactions were significant, there was a trend towards Day affecting groups differentially dependent on whether they had undergone a retrieval session (Day*Reactivation: $F_{1,20} = 4.29$, $p = .052$). Separate analyses of the effect of day in the No Retrieval and Retrieval groups did not reveal a significant effect of Day in either of these groups (No Retrieval: $F_{1,14} = 1.65$, $p = .219$; No retrieval: $F_{1,6} = 3.30$, $p = .119$). There were no significant interactions between Day and Drug in either group (Retrieval: $F_{1,14} = 1.30$, $p = .273$; No Retrieval: $F_{1,6} = 0.89$, $p = .382$).

The latency to reach the 10th lever press did not vary between the FI15 baseline sessions and the 6th day of second-order ($F_{1,20} = 1.82$, $p = .193$; Figure 5.7B). There was, however, a significant interaction between Day and whether animals had undergone a reactivation session ($F_{1,20} = 6.93$, $p = .016$). Separate analysis of Retrieval and No Retrieval groups revealed that Day had affected the latency to make the 10th lever press in the No Retrieval ($F_{1,6} = 12.36$, $p = .013$), but not the Retrieval groups ($F_{1,14} = 1.01$, $p = .331$). Furthermore, in the No Retrieval group there was a significant Day*Drug interaction ($F_{1,6} = 7.77$, $p = .032$), driven by an increase in time from baseline to reach the 10th lever press in MK-801, but not vehicle, treated groups (Figure 5.7B). In contrast, analysis of only the animals that underwent a reactivation session revealed no Day*Drug interaction ($F_{1,14} = 0.89$, $p = .769$). The latency to make the 10th response on the 6th day of second order did not significantly differ between reactivated MK-801 and vehicle groups ($t_{14} = 1.89$, $p = .080$; Figure 5.7B). These latter two results are in stark contrast to the pattern that was observed when the number of lever presses made in the first interval was analysed in a similar fashion (see above).

It appeared that the increase in responding that was seen following the introduction of the second-order schedule of reinforcement was not the result of general, non CS-specific effects as the pattern of responding seen before presentation of this CS did not mirror that of those made in the first interval. The effect of MK-801 treatment at memory reactivation to increase responding in a second-order schedule of reinforcement therefore appeared to be via an enhancement of the CS to potentiate responding, rather than a change in contextual or instrumental associations that could also result in an increase in responding.

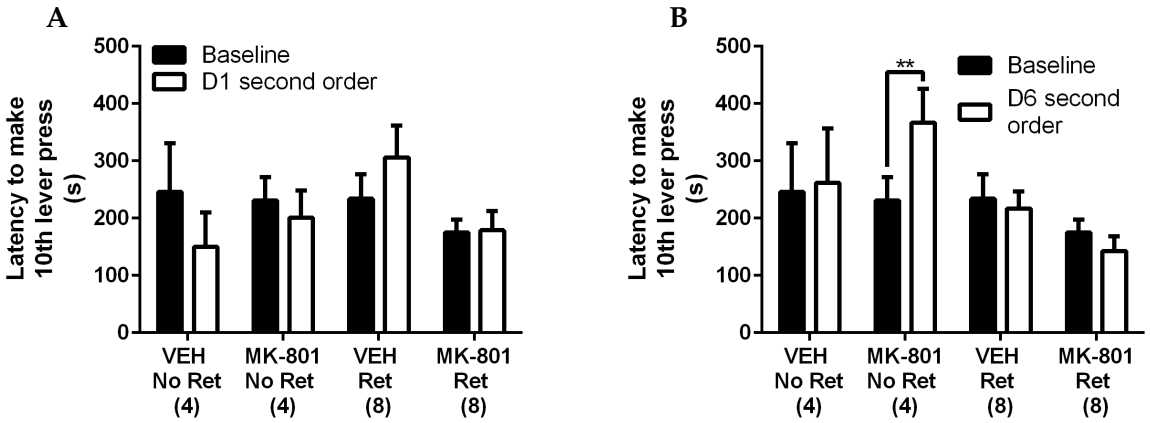


Figure 5.7: Effects of MK-801 treatment before reactivation with non-contingent CS presentation on the latency to make 10 lever presses in a second-order schedule of reinforcement. **A**: Time taken to make the 10th lever press on the 1st day of the second-order training in comparison to a FI15 baseline. **B**: Time taken to make the 10th lever press on the 6th day of the second-order training in comparison to a FI15 baseline. Data are represented as means +SEM. The numbers beneath of the bars represent the N of each group. ** p<.01

Training

All groups acquired the operant response similarly, showing equivalent patterns of responding on the active lever throughout the training sessions, regardless of prospective groupings (all interactions: $F_{3.8,75.9} < 1.31$, $p > .275$; all between-subjects main effects: $F_{1,20} < 0.52$, $p > .480$; Figure 5.8A). All groups took a similar number of days of FR1 to earn 70 CS-US pairings (mean: 4.25, all main effects and interactions: $F_{1,20} < 0.12$, $p > .735$). There were also no significant interactions between any factor in inactive lever pressing across training ($F_{2.4,48.4} < 0.66$, $p > .774$) and no main effects of prospective groups ($F_{1,20} < 0.25$, $p > .624$; Figure 5.8B). Prospective groupings did not affect the total number of CSs earned in training ($F_{1,20} < 0.78$, $p > .388$; Figure 5.8C).

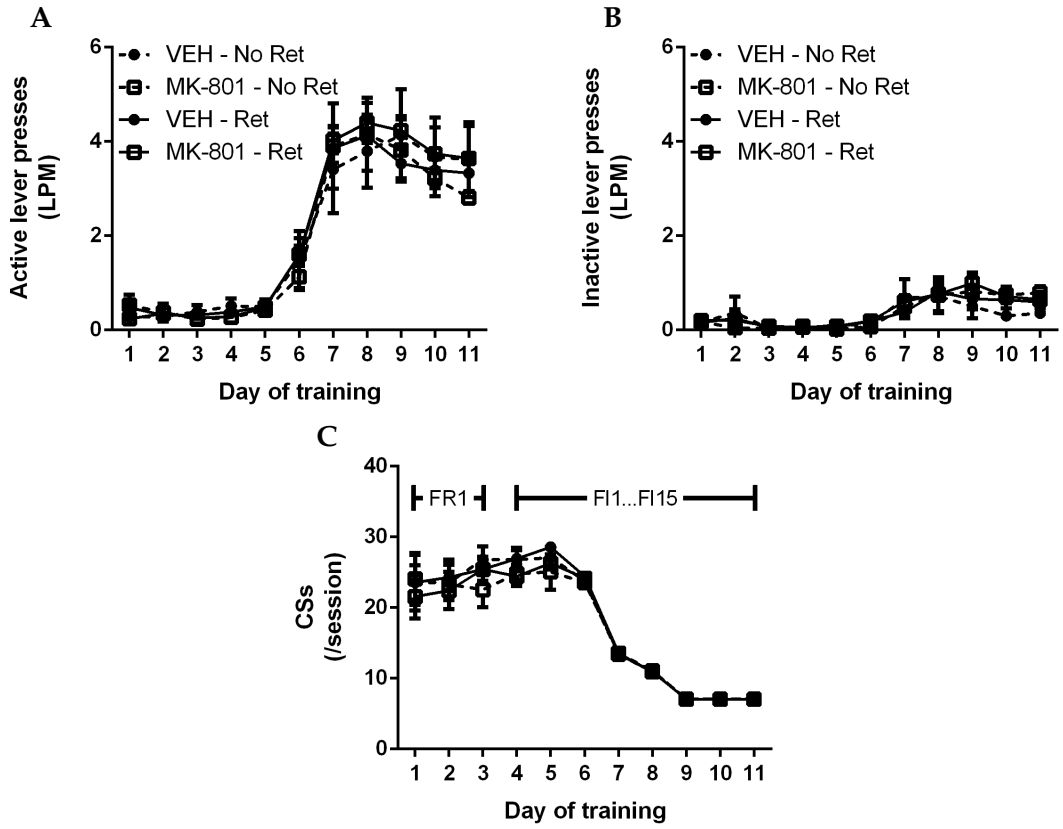


Figure 5.8: Training data for Experiment 3. **A**: Rate of active lever pressing during the last 11 training sessions. See C for schedule of reinforcement. **B**: Rate of inactive lever pressing during the last 11 training sessions. See C for schedule of reinforcement. **C**: Conditioned stimuli earned in the last 11 training sessions. Data are represented as means ± SEM. N=4-8 per group.

Experiment 4: Effects of NMDA receptor antagonism with CPP at cocaine associated memory reactivation with non-contingent CS presentation on subsequent memory expression

After 3 experiments failed to produce an amnestic effect of MK-801 treatment, an alternative NMDA receptor antagonist, CPP, was used. This drug has a greater affinity for the GluN2A subtype of the NMDA receptor (Feng *et al.*, 2004; Feng *et al.*, 2005), activation of which appears to be critical for reconsolidation to take place (Milton *et al.*, 2013). In contrast GluN2B receptor subunits, which MK-801 also targets (Wong *et al.*, 1986; Wong *et al.*, 1988), are required for destabilisation and the action of this drug may have prevented this process in previous experiments, meaning the memory was unable to become reactivated (Ben Mamou *et al.*, 2006; Milton *et al.*, 2013; Yu *et al.*, 2016). This experiment was similar to Experiment 1A, except that CPP was administered before memory reactivation with non-contingent CS exposure instead of MK-801 (see Figure 5.9A). The ability of the drug-paired cue to reinstate responding after instrumental extinction was also assessed.

Test results

CPP administration prior to memory reactivation had no effect on the subsequent ability of the CS to maintain responding in an initial relapse test. This was indicated by animals in both treatment groups making a similar number of active lever presses during this session ($F_{1,14} = 0.14$, $p = .712$; Figure 5.9B). Drug treatment did not affect the number of inactive lever presses made at test ($F_{1,14} = 0.45$, $p = .513$; Figure 5.9D). Splitting the session into 15-minute bins did not reveal any effects of CPP administration on the number of responses made on the active (Bin*Drug: $F_{1.5,21.6} = 0.11$, $p = .849$; Figure 5.9C) or inactive (Bin*Drug: $F_{1.5,21.3} = 0.77$, $p = .443$; Figure 5.9D) lever across the session.

The ability of the CS to reinstate responding after a period of instrumental extinction was not affected by CPP treatment prior to memory reactivation. Animals increased their active lever pressing in response to the CS to a similar degree regardless of drug treatment. This was substantiated by an effect of CS presentation to increase responding ($F_{1,14} = 22.78$, $p < .001$) equally across groups (CS*Drug: $F_{1,14} = 0.06$, $p = .812$; Figure 5.10A). These results therefore further suggested that CPP treatment at reactivation was ineffective at preventing reconsolidation of the cocaine CS-US memory.

Training

The results of the test sessions could not be explained by pre-existing differences in the different drug groups. There were no differences between the prospective groups in the rate of active lever pressing during training (Drug: $F_{1,14} = 0.07$, $p = .794$; Day*Drug: $F_{2.5,35.0} = 0.69$, $p = .542$; Figure 5.11A). The same was true of inactive lever pressing (Drug: $F_{1,14} = 0.11$, $p = .749$; Day*Drug: $F_{1.4,19.1} = 0.19$, $p = .745$; Figure 5.11B). Both groups received a similar number of CS-US pairings during training (Drug: $F_{1,14} = 0.05$, $p = .824$; Figure 5.11C).

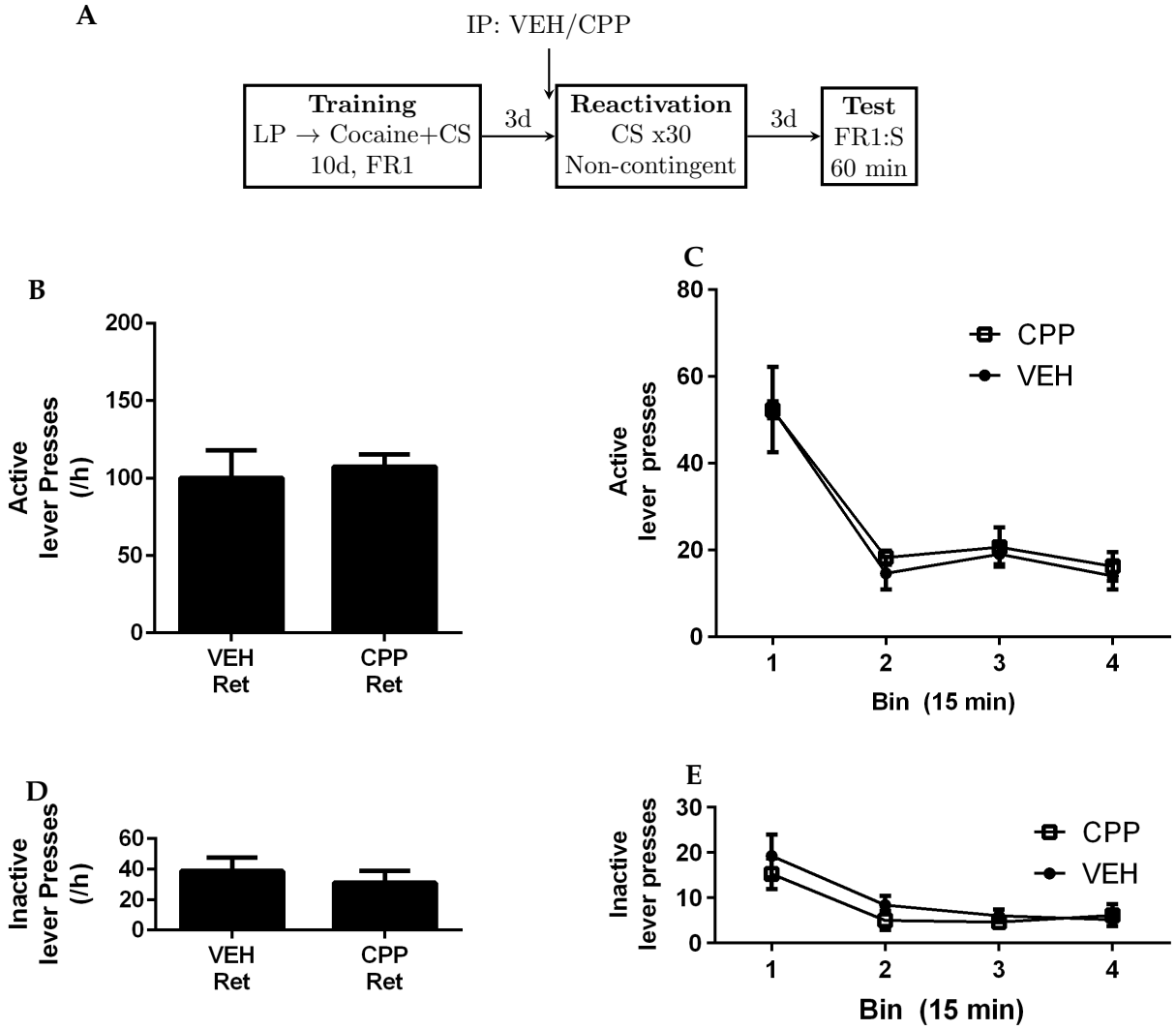


Figure 5.9: CPP administered before reactivation of a memory associated with cocaine using non-contingent CS exposure had no effect on the subsequent ability of this stimulus to maintain responding. **A**: Schematic of experimental procedures in Experiment 4. **B**: Total number of active lever presses made in the test session. **C**: Active lever presses from the test session, divided into 15-minute bins. **D**: Total number of inactive lever presses made in the test session. **E**: Inactive lever presses from the test session, divided into 15-minute bins. Data are represented as means +/±SEM. N=8 for each group.

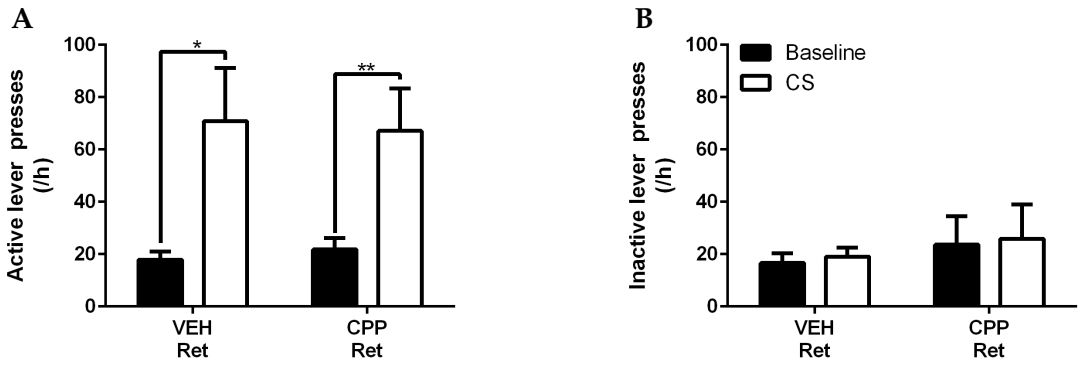


Figure 5.10: Effects of CPP treatment before memory reactivation on subsequent cue-induced reinstatement. **A**: Effect of reintroduction of a cocaine paired CS on responding on the active lever. Baseline refers to responses made on the previous day. **B**: Effect of reintroduction of a cocaine paired CS on responding on the inactive lever. Data are represented as means +SEM. N=8 for each group. * p<.05, ** p<.01

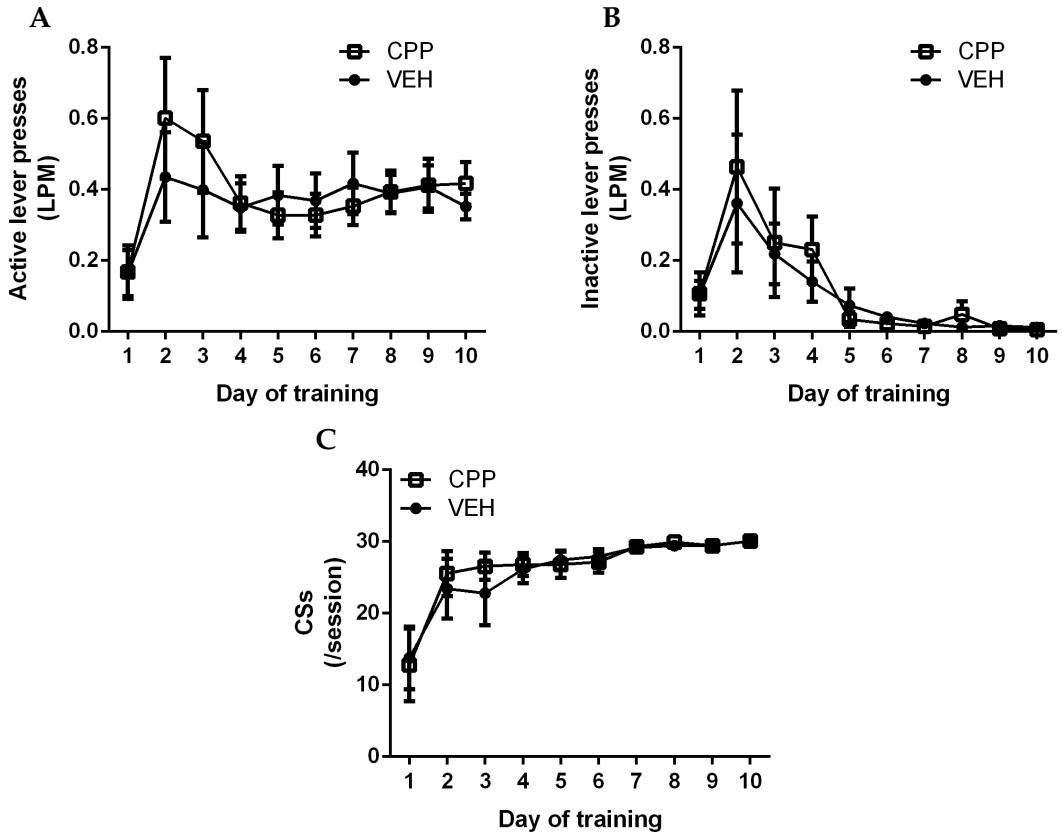


Figure 5.11: Training data for Experiment 4. **A**: Rate of active lever pressing during training sessions. **B**: Rate of inactive lever pressing during training sessions. **C**: Conditioned stimuli earned in the training sessions. Data are represented as means ± SEM. N=8 for each group.

Discussion

Summary of results

Here it was reported that despite using behavioural parameters that have previously been shown to result in memory reconsolidation (Lee *et al.*, 2006a; Milton *et al.*, 2008a), combined with the use of pharmacological compounds known to prevent this process (Lee *et al.*, 2006b; Milton *et al.*, 2008a; Suzuki *et al.*, 2004), it was not possible to reduce responding for a cocaine-paired cue. In contrast, in two of the experiments an increase in responding was reported as a result of this treatment, potentially via a blockade of extinction-like processes and/or an enhancement of reconsolidation. This apparent failure to block memory reconsolidation was despite the use of two different NMDA receptor antagonists, both contingent and non-contingent cue presentation at retrieval, and a range of techniques used to assess rewarding properties of the drug-paired cue. The implications and possible explanations of these data are discussed below.

Relationship to previous work

In order for a memory to become destabilised and subsequently reconsolidated conditions of retrieval must be sufficient for these processes to take place. There are numerous possibilities as to why a given retrieval session might fail to result in memory reactivation, some of which are outlined below. The degree of similarity between training and reactivation sessions is first discussed, followed by the saliency of CS presentation. The particular neurochemical mechanisms required for reconsolidation to take place are then considered, followed by differences between the present studies and those conducted previously and which of these might account for the failure to replicate their findings. Finally, the possible mechanisms for the ability of MK-801 treatment to enhance responding is discussed.

During self-administration the cocaine-paired cue was only ever presented upon an active lever press. In contrast, in the reactivation sessions for Experiment 1a, 3 & 4 the CS was presented non-contingently. Although pavlovian memories formed during cocaine self-administration have previously been reported to destabilise upon non-contingent or contingent cue presentation (Lee *et al.*, 2005b; Lee *et al.*, 2006a; Milton *et al.*, 2008a; Sanchez *et al.*, 2010), previous experiments using food as a reinforcer have demonstrated that only CS presentation that is contingent upon the animal making a response result in memory reactivation (Lee and Everitt, 2008b). With this in mind,

the possible effects of the contingency of CS presentation were investigated in Experiment 1b; the same animals underwent a second reactivation session where the cocaine-paired cue was presented only upon an active lever press. However, this was unsuccessful in leading to a sensitivity of the memory to NMDA receptor antagonism prior to retrieval; in contrast, animals treated with MK-801 increased their lever pressing in a subsequent test session. This suggested that MK-801 was preventing extinction, rather than reconsolidation. It was possible that this was due to some interaction with the previous reactivation session, which may have altered the susceptibility of the memory to become destabilised and/or extinguished.

The possible requirement for contingent CS presentation to result in memory destabilisation was further addressed in Experiment 2, with cue delivery now only occurring upon an active lever press in the initial reactivation session. Once again, this manipulation was apparently not effective in triggering reconsolidation, since animals treated with the NMDA receptor antagonist, MK-801 prior to this modified reactivation session responded at similar levels at test to those treated with vehicle.

One possible hypothesis arising from these data was that the association between the CS and US was not undergoing reconsolidation because of the lack of saliency of the cocaine paired cue. Under an FR1 schedule of reinforcement an animal can 'titrate' to achieve the desired level of cocaine in the bloodstream (e.g. Ahmed and Koob, 1998). Whilst the presentation of the cue does lead to a cocaine infusion, other factors may also come to predict this, including the lever press response and contextual cues of the conditioning chamber. Experiment 3 therefore sought to reduce the impact of these two additional associations, through the introduction of an FI schedule of reinforcement. Here, neither the lever press nor the contextual cues alone can predict the infusion of cocaine – animals will undergo extended periods of lever pressing in the operant box without infusion of cocaine. It is only the illumination of the CS light (and retraction of the levers), that can accurately predict the infusion of cocaine. It was hoped that this would increase the salience of the CS, thus allowing presentation of this stimulus alone to result in reconsolidation. Once again, however, this was not successful.

In three experiments MK-801 treatment in combination with a memory reactivation session failed to produce any amnestic effect. One potential difficulty in using MK-801 to prevent reconsolidation is that it can prevent memory destabilisation (Yu *et al.*, 2016), although here is some evidence that CS-cocaine memories are resistant to these destabilisation-preventing effects, since pre-reactivation

intra-BLA infusion of AP-5 prevents destabilisation of fear (Ben Mamou *et al.*, 2006), but not cocaine-paired memories (Milton *et al.*, 2008a). Because the ability of NMDA receptor antagonists to prevent destabilisation appears to depend on GluN2B subunit activation (Milton *et al.*, 2013) an alternative NMDA receptor antagonist, CPP, was used, which has a slight preference for the GluN2A subunit (Feng *et al.*, 2004; Feng *et al.*, 2005). Treatment with this drug was also ineffective at preventing reconsolidation; administration of CPP before the memory reactivation session, consisting of non-contingent CS presentation, had no impact in subsequent tests of the ability of the CS to maintain responding.

All of the present experiments were based on those that have been conducted previously. However, there were some minor differences between these and the experiments described herein. Whilst Experiment 2 was based on Milton *et al.* (2008a), in this paper training sessions were conducted across 9 days and were an hour in duration, rather than the 2 hour sessions across 10 days in this experiment. The reactivation session also ended once animals received 30 CS presentations, rather than after 15 minutes as used here. Although animals in Experiment 2 were likely to have received more CS-US pairings during training, the number of CSs presented in the reactivation sessions was approximately equal to the Milton *et al.* study. Whilst Experiments 1 and 4 were based on Lee *et al.* (2006a), there were some differences between those and the present experiments. For example, in the equivalent experiment animals in the Lee *et al.* training sessions were 3 hours in duration, resulting in animals in Experiments 1 and 4 receiving fewer pairings. The number of CSs presented at reactivation is a crucial factor in determining whether a memory is destabilised and likely interacts with the extent of training. For example, contextual fear memories that have received more pairings require longer reactivation sessions to trigger reconsolidation (Suzuki *et al.*, 2004). 3 CS presentations results in the destabilisation of a goal-tracking memory after 6, but not 3 or 12 days of training (Reichelt and Lee, 2013a). It is possible, therefore, that the altered proportion of reinforced CSs to CSs in the reactivation session meant the memory did not reconsolidate.

Experiment 3 was modified in order to ensure that animals received the same extent of training as in Lee *et al.* (2006a), with animals in this and the equivalent experiment receiving very similar numbers of reinforced CS presentations. Despite this, treatment with an NMDA receptor antagonist at reactivation did not reduce the subsequent reinforcing properties of the reactivated stimulus. Assuming that the relationship between reinforced and non-reinforced CS presentations required to trigger reconsolidation is consistent between experimenters the memory should have become destabilised in the reactivation session. It is worth noting that not only was there no impairment in the acquisition of second-order in animals treated with MK-801, but in fact responding was increased in these

animals. This could not be attributed to the prevention of extinction, since the memory reactivation procedure did not result in decrease responding, as would be expected if this were the case. At this point it should be highlighted that a notable difference between the Lee *et al.* (2006a) experiment and those conducted here was the intervention to (attempt to) disrupt reconsolidation. The Lee *et al.* paper used intra-BLA infusion of antisense oligodeoxynucleotides (ASOs) to prevent expression of immediate early gene (IEG) *zif-268*, rather than the systemic NMDA receptor antagonism used here. There is a wealth of literature suggesting that broad spectrum NMDA receptor antagonism as a method of preventing memory reconsolidation should be effective, previous studies have shown these are receptors are required for reconsolidation of both discrete (Lee *et al.*, 2006b; Merlo *et al.*, 2015) and contextual (Heath *et al.*, 2015; Suzuki *et al.*, 2004) fear associations, appetitive pavlovian associations for both natural (Lee and Everitt, 2008b; Lee and Everitt, 2008c; Reichelt and Lee, 2013a) and drug reinforcers (Milton *et al.*, 2008a) and more recently, instrumental associations for both food and drug reinforcers (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014), alongside their ability to prevent increases in the expression *zif-268* in the BLA (Milton *et al.*, 2008a), known to be critical in the reconsolidation of these associations (Lee *et al.*, 2005a). Furthermore, meta-analysis of numerous studies using NMDA receptor antagonism to prevent reconsolidation concluded there was a robust impairment in this process for reward-related memories with this drug (Das *et al.*, 2013). Theoretically, therefore, if reconsolidation was taking place in the reactivation sessions NMDA receptor antagonism should have been effective at preventing it.

The data from Experiment 3 suggested that reconsolidation was enhanced by MK-801 treatment. There are several published examples of enhancing reconsolidation of both aversive (Bredy and Barad, 2008; Lee *et al.*, 2006b) and appetitive (Lee *et al.*, 2009; Schramm *et al.*, 2016) pavlovian associations. However, typically these effects are achieved with manipulations which would be expected to enhance plasticity, rather than NMDA receptor antagonism, that would be expected to prevent it. Whilst the majority of published reports suggest that NMDA receptor antagonism results in impairments in both consolidation and reconsolidation, there is also some evidence that it can have an enhancing effect on each of these processes. Administration of high doses of MK-801 before cued fear conditioning sessions can enhance acquisition (Gould *et al.*, 2002) and pre-reactivation treatment of the same drug (at lower doses) can result in increased fear to a shock-paired context (Flavell, 2015). Although the mechanism for these enhancements is unclear, one possibility is that they are the result of the non-specific effects of MK-801. Alongside antagonism of NMDA receptors, this drug also increases dopamine and serotonin metabolite levels across several regions of the brain (Löscher *et al.*, 1991), increases Fos expression (Sonnenberg *et al.*, 1989), inhibits activity of nicotinic

acetylcholine receptors (Amador and Dani, 1991) and increase acetylcholine levels (Hasegawa *et al.*, 1993).

Another possibility is that the manipulation disrupted one aspect of the excitatory memory trace, but not another. Recent investigations have shown that after limited training, responding in a second-order schedule of reinforcement is BLA-dependent, but after extended training relies upon the central nucleus of the amygdala (CEN) (Murray *et al.*, 2015). One possibility is that during the reactivation session the BLA, but not CEN, dependent trace became reactivated. Reconsolidation of the BLA trace was then prevented, leaving only CEN-dependent responding. Given that responding requiring the CEN is hypothesised to be habitual and characterised by high response levels, this could explain the increased responding in the MK-801 treated animals. Post-training lesions of the BLA have been shown to result in an increase in responding in a second-order schedule of reinforcement for cocaine and an enhancement in second-order conditioning for food (Holland, 2016; Murray, *unpublished observations*). This would also explain why the increase in responding was only seen several sessions after memory reactivation – as animals have more experience with the second-order schedule of reinforcement brain regions associated with habitual responding, i.e. the CEN, will start to become recruited. Future investigations could explore this possibility through inactivation of the CEN after the reactivation sessions – it would be predicted that MK-801 treatment prior to memory reactivation would result in a heightened to sensitivity to this treatment in comparison to vehicle treated controls.

Finally, one possibility is that the effects were unrelated to CS presentation, but the result of disrupted instrumental memory reconsolidation. However, this is unlikely for several reasons. Analysis of the latency to reach the 10th lever press in the second-order sessions, a potential measure of CS independent responding, was unaffected by drug-treatment at reactivation. Furthermore, the reactivation session consisted of only non-contingent CS presentation; there was no opportunity to reactivate the instrumental response. In order to rule out this possibility, it would have been necessary to conduct test sessions in the absence of CS presentation and examine whether the effect of MK-801 at reactivation to increase responding persists.

Implications for subsequent research

It was not possible to determine the parameters that result in the destabilisation of a pavlovian memory associated with cocaine. These experiments were all conducted in animals that have been trained to an extent that has previously been suggested to a reflect a casual user (Murray *et al.*,

2012), a protocol that does not model habitual nature of drug seeking. The inability to disrupt reconsolidation in these associations does not, however, preclude investigation of reconsolidation of reward associated memories that have undergone extensive training, the primary aim of the following chapter.

Chapter 6: Breaking a habit: disruption of memory reconsolidation after extensive training

Introduction

Experiments in Chapter 5 were unable to disrupt reconsolidation of a pavlovian memory formed during cocaine self-administration. Specifically, animals that had undergone approximately 10 days of training showed no reduction in cocaine seeking after being administered an N-methyl-D-aspartate (NMDA) receptor antagonist prior to a memory retrieval session. This was despite attempts to increase the likelihood of reconsolidation taking place through modifications of the re-activation session and the use of different pharmacological compounds used to attempt to block this process. A variety of different methods were also used to assess the integrity of conditioned stimulus (CS)-unconditioned stimulus (US) association. Whilst these results may suggest there is limited potential for reconsolidation-based treatments for drug addiction it is worth noting that these experiments were carried out on animals that had received relatively few days of training and were therefore likely more reflective of a 'casual user' rather than an addicted individual. However, it is the memories underlying well-established, habitual behaviour that will be the targets in reconsolidation-based treatments for disorders such as drug addiction. It is known that strong (Wang *et al.*, 2009), old (Inda *et al.*, 2011; Milekic and Alberini, 2002; Suzuki *et al.*, 2004) memories are more resistant to destabilisation, potentially making disrupting the reconsolidation of habitual memories more difficult than those have undergone limited training. However extensive training may also present the opportunity to induce a greater prediction error (PE), a key factor in determining whether a given retrieval trial results in reactivation of the memory (Pedreira *et al.*, 2004; Reichelt and Lee, 2013c; Sevenster *et al.*, 2013). Furthermore, the distinct neurocircuitry supporting habitual behaviour (e.g. Murray *et al.*, 2012; Murray *et al.*, 2015) may mean that the issues in the previous chapters that prevented memory destabilisation may not necessarily preclude blocking reconsolidation of these memories. This chapter therefore attempted to disrupt well-established

appetitive memories, with the view that it is these associations that will be the target for future treatments of psychiatric disorders including drug addiction and potentially obesity.

Whilst responses for food rewards are initially governed by action-outcome (A-O) associations, as training progresses these become driven by stimulus-response (S-R) pairings. The neural structures required for these two types of responding have received extensive attention. The dorsomedial striatum (DMS), prelimbic (PL) cortex, basolateral amygdala (BLA) and mediodorsal thalamus are required for goal-directed responding (Corbit *et al.*, 2003; Killcross and Coutureau, 2003; Lingawi and Balleine, 2012; Yin *et al.*, 2005b). In contrast, the dorsolateral striatum (DLS), infralimbic (IL) cortex and central nucleus of the amygdala (CEN) appear to have an exclusive role in S-R dependent responding (Coutureau and Killcross, 2003; Lingawi and Balleine, 2012; Yin *et al.*, 2004). The shift in the requirements for these brain regions in these two types of responding has been demonstrated by a change in the sensitivity of responding to reinforcer devaluation. Lesions or inactivation of brain structures associated with A-O responding result in an insensitivity to these procedures, whilst the same manipulations in regions required for S-R responding result in a restored susceptibility to outcome devaluation in habitual animals. The ability to reinstate goal-directed responding in animals that were once habitual suggests A-O and S-R associations are formed in parallel, with one association dominating control over behaviour depending on (typically) the extent of training.

The neural structures underlying responding augmented by cocaine-paired CSs have been shown to undergo a shift in their neural basis depending on the extent of training, in an apparent parallel to the shift in goal-directed to habitual instrumental responding. Whilst cocaine seeking at an early stage of training depends upon activation of the DMS and BLA (Murray *et al.*, 2012; Murray *et al.*, 2015), after extended periods of drug-seeking these responses become dependent on the DLS and CEN (Belin and Everitt, 2008; Murray *et al.*, 2012; Murray *et al.*, 2015; Vanderschuren *et al.*, 2005). Whilst these studies did not specifically probe whether this responding was habitual, the reliance of this behaviour upon similar neural mechanisms suggests this may be the case. Furthermore, procedures that do permit the testing of whether drug-seeking is goal-directed or habitual have shown that the DLS is recruited as responding for these rewards becomes insensitive to the value of its outcome (Corbit *et al.*, 2012; Zapata *et al.*, 2010). Whilst in a much earlier stage of investigation, it appears that after extensive training cue-dependent food-seeking responses for highly palatable food also become dependent on the DLS. Infusion of the dopamine receptor antagonist α-flupenthixol into the DLS results in a reduction in such behaviours (Giuliano *et al.*, in preparation), whilst this manipulation is without effect on food-seeking responses after limited training (Belin and Everitt, 2008).

The majority of works conducted to date on appetitive memory reconsolidation have used training protocols that are relatively short in duration and would be unlikely to recruit the DLS and CEN (e.g. Flavell and Lee, 2013; Lee *et al.*, 2006a; Milton *et al.*, 2008a; Sanchez *et al.*, 2010). The reliance of responding that has been extensively trained upon these regions may affect the ability of these memories to reconsolidate. For example, cell-firing in the CEN is more GluN2B dependent than that of the BLA (Sah and De Armentia, 2003) and studies in fear memory reconsolidation have demonstrated that destabilisation is dependent upon activation of these receptors (Milton *et al.*, 2013), suggesting that a change in the dependence of the subunit activity between the BLA and CEN may affect the ability of memories dependent on these regions (Murray *et al.*, 2015) to destabilise.

The extent of training is not the sole determinant in the transition from goal-directed to habitual responding; this is also influenced by the schedule by which responses are reinforced. Whilst ratio schedules promote the formation of goal-directed responding, interval schedules will more likely result in habitual behaviour (Dickinson *et al.*, 1983). The nature of ratio schedules (where a certain number of responses are required to obtain reward) mean that as the rate of responding increases, so does the reinforcement rate, promoting the formation of A-O associations. The opposite is true of interval schedules (where a response is reinforced based on the time since last reward delivery), where at high rates of responding the correlation between response and reinforcement rate is very low, resulting autonomous responding whereby the instrumental response becomes separated from the reinforcer it produces (S-R responding) (Dickinson, 1985). The shift in cocaine-seeking from being DMS to DLS dependent is similarly affected by reinforcement schedule. Fixed ratio (FR) schedules of reinforcement result in the formation of drug seeking responses that remain dependent on the DMS, apparently regardless of the extent of training. In contrast, when responding is reinforced on a second-order schedule of reinforcement the DLS is recruited after the same number of days of training (Murray *et al.*, 2012). In these schedules animals are required to respond for long (15-20 minutes) periods in the absence of primary reinforcement. However, during these intervals responses result in the presentation of the reward-paired CSs; delivery of which results in a dramatic increase in the rate of responding (Arroyo *et al.*, 1998; Everitt and Robbins, 2000).

Two factors appear to govern the transition from goal-directed to habitual reward seeking: the use of second-order order schedules of reinforcement and the extent of training. Both of these characteristics may mean that memories underlying habitual responding are resistant to reconsolidation.

Inherent in second-order schedules of reinforcement is that the CS is presented multiple times without reward delivery. One of the key factors in determining whether a retrieval session will

result in memory destabilisation is the presence of PE (e.g. Pedreira *et al.*, 2004; Reichelt and Lee, 2013c; Sevenster *et al.*, 2013), that is, whether the reinforcer is delivered as expected. It is possible that the repeated experience of CS presentation in the absence of reward in the second-order training sessions means that reactivation sessions, which typically also consist of delivery of CS without reinforcer delivery (e.g. Lee *et al.*, 2006a; Milton *et al.*, 2008a; Sanchez *et al.*, 2010), will not trigger sufficient PE to result in destabilisation. Alternatively, the use of second-order schedules, combined with extensive training, may in fact present an opportunity to increase the PE at reactivation. Responding during the first interval in these schedules, which occurs in the absence of drug delivery, 'scallops', with the rate of lever pressing increasing as the fixed interval (FI) comes closer to completion (Arroyo *et al.*, 1998), perhaps indicative of the anticipation of reward. Violation of this expectancy should result in a large PE signal perhaps leading to memory destabilisation.

Whilst extensive training should lead to an increase in the likelihood of a given trial to result in PE, this does not necessarily mean these memories are more likely to destabilise. Fear memories that have undergone extensive training are more resistant to the effects of post-reactivation anisomycin (Wang *et al.*, 2009). In some cases these difficulties can be overcome with longer reactivation sessions (Suzuki *et al.*, 2004) and stronger memories may become more receptive to disruption of reconsolidation with repeated reactivation sessions (Robinson and Franklin, 2010). Furthermore, in some cases memory strength does not appear to preclude or affect destabilisation; propranolol is equally effective at preventing reconsolidation of a cued fear memory that has undergone 2 or 5 pairings (Taherian *et al.*, 2014). Moreover, the naturally occurring compound Garcinia is able to prevent reconsolidation of CS-drug memories formed across 12 or 24 days of self-administration (Monsey *et al.*, 2017).

Three experiments in this chapter investigated the reconsolidation of memories underlying responding after extensive training in a second-order schedule of reinforcement for both cocaine (Experiment 1) and food (Experiment 2 & 3). Animals were trained in a similar fashion as previous reports showing DLS-dependent responding (Belin and Everitt, 2008; Giuliano *et al.*, in preparation). The reactivation procedure used was designed to maximise PE through the violation of several learned expectancies. Firstly, unlike training, responses during this session did not produce delivery of the CS. Secondly, at the end of the first interval no reinforcer was delivered. Finally, the session ended after this first interval, unlike training where animals continued to respond for rewards after the first reinforcer delivery. In Experiment 3 animals underwent multiple (3) reactivations in order to investigate whether this results in an increased propensity of the memory to

destabilise. As in previous chapters, animals were administered with the NMDA receptor antagonist MK-801 to disrupt reconsolidation in these sessions.

Methods

Animals were extensively trained to respond on a second-order schedule of reinforcement for delivery of cocaine (Experiment 1) or chocolate-flavoured food pellets (Experiments 2 & 3). The effects of NMDA receptor antagonism during memory reactivation on subsequent responding in the same second-order schedule of reinforcement were then assessed.

Procedures were conducted as in General methods except where stated.

Cocaine self-administration experiments

Animals and housing

Animals were 24 male Lister-Hooded rats weighing 300-350g at the time of surgery. 4 days before training began animals were food-restricted and fed 20g of standard rat chow at the end of each day.

Surgery

Intravenous catherisation surgery was conducted as in General methods, with anaesthesia maintained with inhalation of isoflurane mixed with 100% oxygen.

Training procedures

Responses on the active lever were first reinforced under an FR1 schedule of reinforcement for 6d. Animals then progressed through gradually longer FI schedules of reinforcement, beginning at FI1 (min), advancing through 2, 4, 6, 10 for one day each and stabilising for FI15 for a final three days (Belin and Everitt, 2008; Lee *et al.*, 2006a). A second, inactive, lever was present throughout training, responses on which were without consequence.

After pre-training animals were trained under a second-order schedule of reinforcement. Animals first underwent a total of 7 of these sessions. However, due to low levels of responding in the first interval, all animals underwent a further 3d of 'remedial' FR1 with the view that may increase

the ability of the cocaine paired CS to potentiate responding. Starting the day after these sessions the second-order schedule of reinforcement was reintroduced for a further 14d, meaning animals underwent a total of 21d of second-order training. This extent of training has previously shown to result in recruitment of DLS-dependent responding (Murray *et al.*, 2012; Peña-Oliver *et al.*, in preparation).

Reactivation

Reactivation took place the day after completion of second-order training. For this session both levers were presented for 15 minutes but responding was without consequence. Once the 15 minutes had elapsed the next active lever press resulted in illumination of the CS light, de-illumination of the houselight and delivery of 0.1ml of saline. Following saline infusion and CS presentation the sessions terminated and the animal was removed. The reactivation session was different from the prior training sessions in three ways: an abbreviated CS was not presented contingent upon responding, cocaine was not received at the end of the 15 minute interval and the session ended after approximately 15, rather than 75 minutes. 30 minutes before this session animals were either injected with MK-801 (Sigma-Aldrich) or its vehicle.

Test

The test session was carried out exactly as in second-order training and took place 3d after reactivation. This delay between sessions was introduced as this has previously been demonstrated to result an increase in responding (Peña-Oliver *et al.*, in preparation), potentially making a decrease in drug-seeking easier to detect.

Food experiments

Animals and housing

Subjects were 60 male Lister-Hooded rats weighing 240-430g at the start of experiments. The day before training began animals were food-restricted and fed 18-20g of standard rat chow at the end of each day.

Training procedures

After two sessions of pavlovian magazine training, where a light CS preceded reward delivery in a non response-contingent fashion, animals underwent 3-6d of instrumental training, where responses on one of two levers presented resulted in the delivery of chocolate-flavoured sucrose pellets (AIN-76A, Testdiet, IN, USA) on an FR1 schedule of reinforcement. A second, inactive, lever was present throughout training, responses on which were without consequence. Following the FR1 phase an FI schedule of reinforcement was introduced, progressing through FI1, 2, 4, 6, 8, 10 and stabilising for FI15 for a final three days. As the interval increased, so did the number of pellets delivered, with a total of 20 pellets being delivered in the FI15 sessions (adapted from Giuliano *et al.*, 2012).

Following pre-training sessions a second-order schedule of reinforcement was introduced for 21 consecutive sessions, a procedure that has previously been shown to result in responding that is dependent on the DLS (Giuliano *et al.*, in preparation).

Memory reactivation and test

Reactivation sessions were conducted in a similar fashion to Experiment 1. Both levers were presented for 15 minutes and responding was without consequence. After this period had elapsed the next active lever press resulted in illumination of the CS light, de-illumination of the houselight, retraction of both of the levers and delivery of 20 pellets into a metallic dish located outside of the operant chamber, but within the sound-attenuating shell. Following mock pellet delivery and CS presentation the sessions ended and the animal was removed. In each experiment, approximately half of the subjects were treated with MK-801 prior to the reactivation session(s) and the other half treated with vehicle. The number of reactivation sessions that animals underwent varied between experiments.

Animals underwent two test sessions, the first of which was conducted the day after the (final) reactivation session. Test sessions were conducted exactly as the second-order training sessions, with the pellets being delivered into the magazine at the end of each interval. The number of days between each of the second training sessions varied between experiments (see below)

Experiment 2: The effects of MK-801 treatment prior to reactivation of a well-established food-seeking memory

The results of this experiment are represented as combined data for two separate replications, one of which was intended as a pilot investigation. Although there were several differences in the way that these two experiments were conducted, both squads behaved very similarly. Animals in the first time the study was conducted (final n=11, referred to as Squad A hereafter) had previously been used in a fear-conditioning experiment, whilst animals in the second were experimentally naïve (n=18, referred to as Squad B hereafter). Animals in Squad A underwent 3 or 6d of FR1 training, before proceeding through the interval and second-order schedules of reinforcement, whilst animals in Squad B all underwent 6. The second-order training, reactivation and the first test sessions were conducted in the same fashion for both squads, with each of these occurring on consecutive days. The second test session occurred the day after the first in Squad A, and 7d after in the second in Squad B. None of these factors appeared to influence responding in any way.

Experiment 3: MK-801 prior to multiple reactivation sessions of a well-established food-seeking memory

All animals in this experiment received a total of 6d of FR1 before the introduction of interval schedules of reinforcement. Test sessions took place the day after the reactivation session and one week later. The key difference between this and the previous experiment was that the reactivation session (and preceding drug administration) was conducted three times, each on consecutive days.

Statistical analyses

Response rates for the food second-order experiments were much higher than in previous chapters. Because variance in lever pressing tends to increase with the mean (Dickinson and Dawson, 1987), these high rates of responding caused data to become highly non-normal. Normality was assessed with Kolmogorov-Smirnov (K-S) test and if any of the sessions analysed had a non-normal distribution responses data were square root transformed (Dickinson and Dawson, 1987). Normality tests were conducted on each of the following groups of data and if any dataset, from either treatment group were not normally distributed the data from all groups in these sessions were transformed: first and subsequent interval(s) of baseline and test sessions, responding in the reactivation session, first and subsequent intervals of second-order training sessions and response rates during the training sessions conducted before introduction of the second-order schedule of reinforcement. In

cases where multiple K-S values suggested the data were not normally distributed the smallest of these values is reported in the text.

Once assessed for normality and transformed as necessary, data were analysed as in previous chapters. Responding in the test sessions was compared to baseline second-order performance using a mixed-design analysis of variance (ANOVA) with Session as a repeated-measures factor and Drug as a between-subjects factor. Baseline rates of responding were calculated from the two sessions of second-order training before reactivation took place. Any pre-existing differences in the acquisition of the second-order schedules were assessed with Session coded as a within-subjects and Drug as a between-subjects factor. Differences in the drug groups in the rate of responding in the reactivation session(s) were assessed with between-subjects t-tests in Experiments 1 & 2 and with Day coded as a within-subjects factor and Drug as between-subjects in an ANOVA.

Results

Experiment 1: NMDA receptor antagonism during retrieval of a well-established cocaine seeking memory has no effect on subsequent drug seeking

In this experiment animals were trained on a second-order schedule of reinforcement for the delivery of cocaine. After extensive experience on this schedule animals underwent a memory-reactivation session, preceded by treatment with the NMDA receptor antagonist MK-801. Animals were then returned the second-order schedule of reinforcement in order to investigate the impact on this treatment on subsequent responding (see Figure 6.1A).

6 animals were excluded from the experiment owing to illness or blocked or damaged catheters.

Test results

All data from the test session met the requirements for normality, so the raw values were used for analysis ($D_9 < 0.27$, $p > .055$).

There was no evidence that MK-801 treatment prior to reactivation of a well-trained cocaine seeking response had any impact on its reconsolidation, as indicated by very similar levels of responding in the first interval of the test session by both drug treatment groups (Figure 6.1B). Whilst responses in the first interval were increased in comparison to baseline levels of responding ($F_{1,16} = 11.98$,

$p = .003$), this increase was similar in drug treatment groups (Session*Drug: $F_{1,16} = 0.87$, $p = .366$). Drug treatment also had no overall effect on responding ($F_{1,16} = 0.70$, $p = .414$).

Similar results were obtained when responding in the later intervals, after the delivery of cocaine, was analysed. Prior treatment with MK-801 had no impact on responding in these intervals, which did not vary between baseline and test sessions (Figure 6.1C). This was indicated by no effect of Session ($F_{1,16} = 1.35$, $p = .263$), no Drug*Session interaction ($F_{1,16} = 1.04$, $p = .323$) and no overall effect of Drug ($F_{1,16} = 1.00$, $p = .332$).

The number of inactive lever presses did not differ between sessions or drug groups and this was equally true in the first and subsequent intervals. When data from the first interval were analysed there was no effect of Day ($F_{1,16} = 0.04$, $p = .840$), no Day*Drug interaction ($F_{1,16} = 0.84$, $p = .374$) and no overall effect of Drug ($F_{1,16} = 2.19$, $p = .159$; Table 6.1). The same was true when the average number of lever presses for the subsequent intervals was analysed (Day: $F_{1,16} = 1.08$, $p = 0.314$; Day*Drug: $F_{1,16} = 0.27$, $p = .613$; Drug: $F_{1,16} = 1.89$, $p = .188$; Table 6.1).

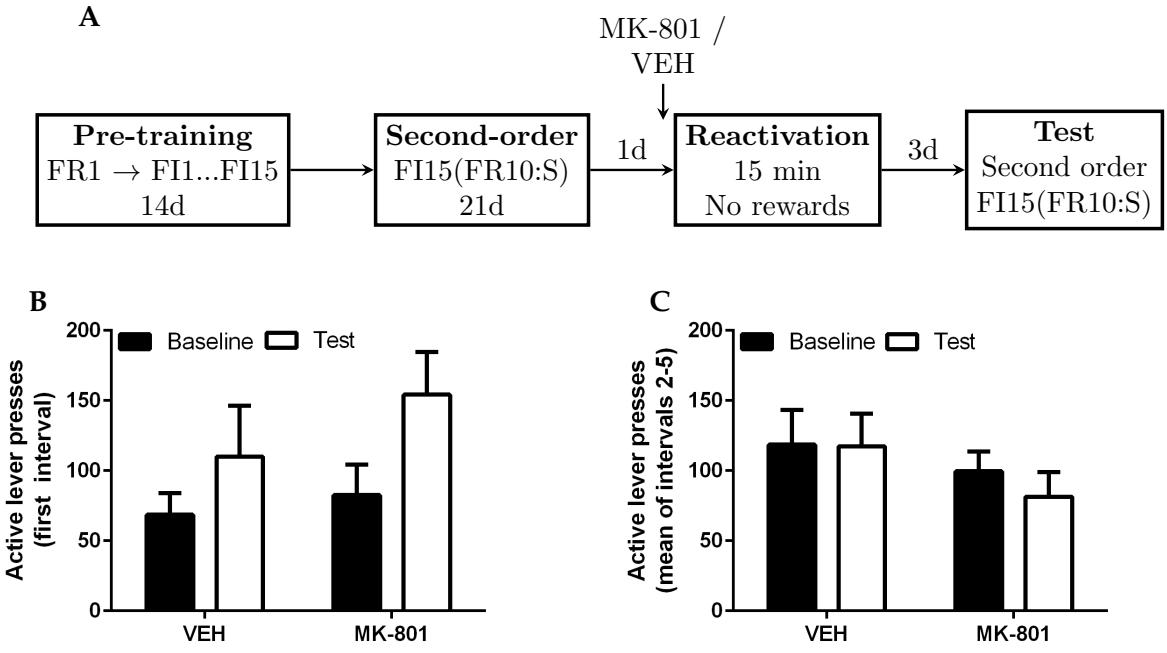


Figure 6.1: MK-801 administered before retrieval of a cocaine seeking memory has no effect on subsequent responding. **A**: Schematic of experimental procedures in Experiment 1. Not depicted in the figure are 3d of additional FR1 training which were conducted after 7d of second-order training. **B**: Active lever presses made in the first interval of the final session of second order and subsequent test sessions. **C**: Active lever presses made in the intervals 2-5 of the final session of second order and subsequent test sessions. Data are represented as means +SEM. N=9 for both treatment groups.

Interval Group	Baseline	Test
1		
Vehicle	3.8 ± 1.58	4.9 ± 2.07
MK-801	10.6 ± 3.97	8.9 ± 2.86
2-5		
Vehicle	5.0 ± 1.16	5.8 ± 1.97
MK-801	9.4 ± 3.14	11.8 ± 4.20

Table 6.1: Inactive lever press data from baseline and test sessions of Experiment 1. Data are presented as means $\pm$ SEM to 2 decimal points.

Training and reactivation

The prospective treatment groups did not differ in their rate of responding on the active lever during the pre-training sessions. Normality analysis of the training sessions conducted prior to the introduction of the second-order schedule of reinforcement revealed that data from several of these sessions were not normally distributed in both treatment groups ($D_9 > 0.29$, $p < .034$) and these data were transformed before being analysed for group differences. Whilst responding varied between sessions ($F_{2.7,42.8} = 111.66$, $p < .001$), both groups showed similar patterns of acquisition (Day*Drug: $F_{2.7,42.8} = 0.72$, $p = .528$) and overall response rates in these sessions (Drug: $F_{1,16} = 0.44$, $p = .517$; Figure 6.2A).

The rate of inactive lever pressing in the pre-training was similar regardless of future drug treatment (Figure 6.2B). Responses were transformed because data from several of these session violated the assumptions of normality in both groups ($D_9 > 0.28$, $p < .038$). Whilst the day of training affected the rate of inactive lever pressing ($F_{13,208} = 23.72$, $p < .001$) this effect was similar between treatment groups (Day*Drug: $F_{13,208} = 0.55$, $p = .893$). Prospective groupings did not influence the overall level of responding on the inactive lever during these sessions (Drug: $F_{1,16} = 0.06$, $p = .817$).

None of the results from the test session could be explained by differences in the number of active lever presses made during second-order training sessions between prospective treatment groups in either the first or subsequent (Figure 6.2C and 6.2D). Data from several of the second-order training sessions were not normally distributed in the first or subsequent intervals ($D_9 > 0.28$, $p < .049$) and as such these data were transformed before statistical analysis took place. The number of responses made in the first interval varied between sessions ($F_{20,320} = 2.54$, $p < .001$) but this change was similar between prospective treatment groups (Session*Drug: $F_{20,320} = 0.59$, $p = .918$) and the overall number of responses made in these sessions did not vary as a function of future group

allocation (Drug: $F_{1,16} = 0.36$, $p = .557$; Figure 6.2C). The same was true of subsequent intervals in these training sessions (Session: $F_{20,320} = 1.91$, $p = .011$, Session*Drug: $F_{20,320} = 0.55$, $p = .942$, Drug: $F_{1,16} = 0.68$, $p = .421$; Figure 6.2D).

The lack of pre-existing differences in the rate of active lever pressing was mirrored in the inactive lever press data (Figure 6.2E and 6.2F). Data from both first ($D_9 > 0.30$, $p < .028$) and subsequent ($D_9 > 0.28$, $p < .049$) intervals violated the assumptions of normality for both treatment groups so were transformed. Whilst Day affected the total number of inactive lever presses made in the first ($F_{20,320} = 2.30$, $p = .001$) and following intervals ($F_{20,320} = 4.81$, $p < .001$), this effect did not vary between treatment groups (Day*Drug, first interval: $F_{20,320} = 0.67$, $p = .857$; subsequent intervals: $F_{20,320} = 0.98$, $p = .482$). There was a non-significant trend for animals that would go on to be treated with MK-801 to respond less on the inactive lever in the first ($F_{1,16} = 3.37$, $p = .085$; Figure 6.2E) but not following intervals ($F_{1,16} = 1.33$, $p = .265$; Figure 6.2F).

Both treatment groups made similar numbers of responses on both levers during the reactivation sessions. Data from vehicle treated rats did not meet normality requirements for either active or inactive lever presses ($D_9 > 0.37$, $p < .001$) so the data were transformed for analysis. Prior drug treatment did not affect the total number of active ($t_{16} = 0.24$, $p = .817$; Figure 6.2G) or inactive lever presses ($t_{16} = 1.95$, $p = .069$; Figure 6.2H).

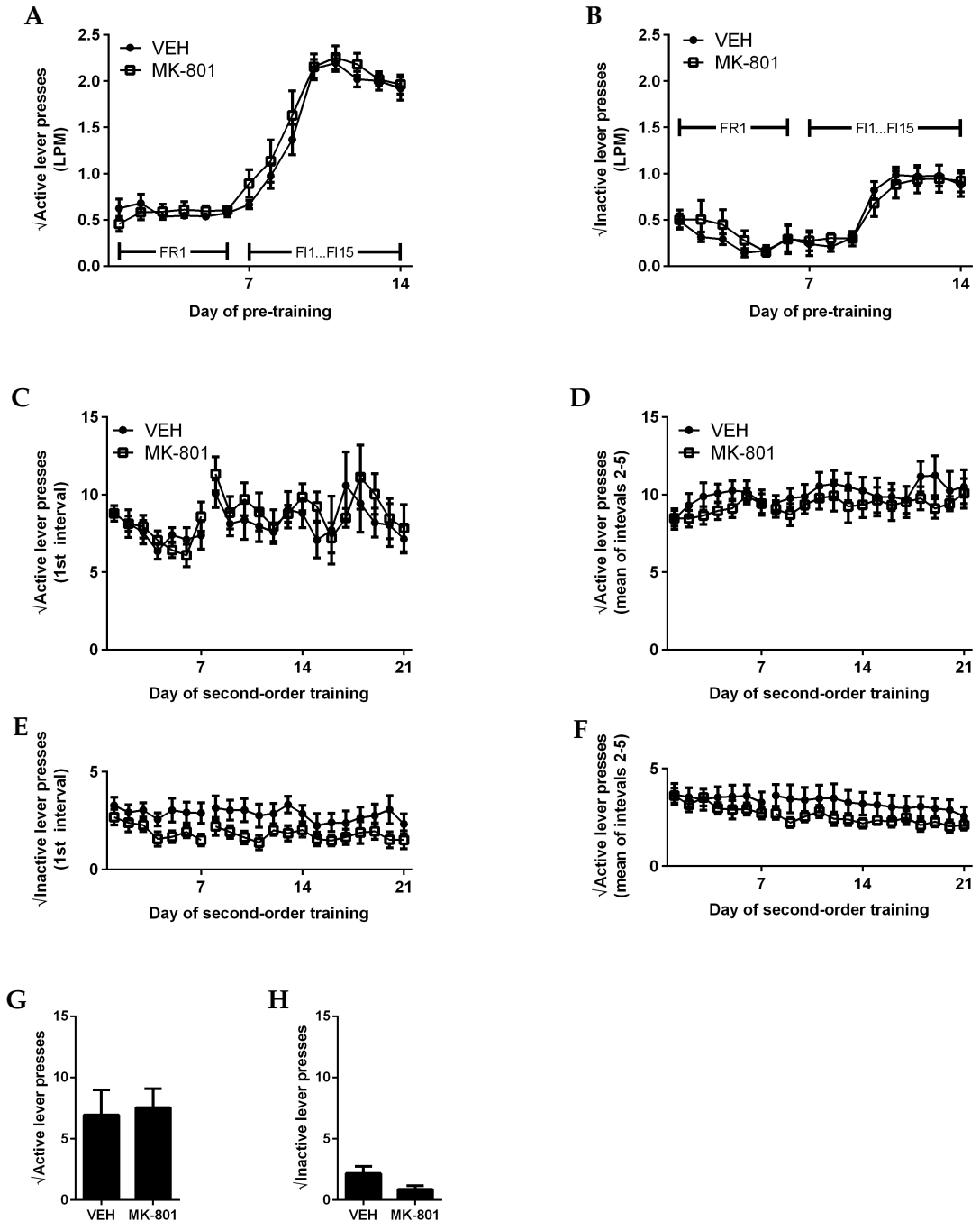


Figure 6.2: Pre-training, second-order training and reactivation data for Experiment 1. **A**: Rate of active lever pressing during pre-training sessions. **B**: Rate of inactive lever pressing during pre-training sessions. **C**: Number of active lever presses made in the first interval during second-order training sessions. The break in the line reflects where animals underwent 3 days of FR1 training. **D**: Number of active lever presses made in intervals 2-5 during second-order training sessions. **E**: Number of inactive lever presses made in the first interval during second-order training sessions. **F**: Number of inactive lever presses made in intervals 2-5 during second-order training sessions. **G**: Active lever presses made in the reactivation session. **H**: Inactive lever presses made in the reactivation session. Data are represented as means +/±SEM. N=9 for both groups.

Experiment 2: NMDA receptor antagonism during retrieval of a well-established food-seeking memory results in a transient decrease in subsequent seeking

This experiment was similar to Experiment 1, except that animals were trained to respond for a food, rather than a drug reward. Animals underwent two test sessions in order to investigate the effect of MK-801 treatment at reactivation (see Figure 6.3A).

One animal was excluded from this experiment after it was observed having multiple seizures.

Test results

Administration of MK-801 prior to the reactivation session resulted in a decrease in responding from baseline levels when tested under drug free conditions the next day (Figure 6.3B). However, this treatment did not affect the number of responses made in the second interval, following reward delivery (Figure 6.3C). It appeared that the loss of the apparent amnestic effect by the second interval was the result of having being reminded of the association between the CS and the reward, rather than animals having recently come into contact with the reinforcer, since MK-801 treatment also had no effect on responding in the first interval of a second test session (Figure 6.3B). The analysis supporting this pattern of results is reported below.

Data from the first interval of the baseline session of second-order and two test sessions were not normally distributed in the MK-801 treated group ($D_{15} > 0.34$, $p < .001$) and as such all the data from these sessions was transformed (data from the baseline sessions also approached the threshold for violating normality in the vehicle treated group: $D_{14} = 0.22$, $p = .057$). Baseline responding in the second interval was not normally distributed in all treatment groups ($D_{15} > 0.23$, $p < .030$) and for the first test in the MK-801 treated group ($D_{15} = 0.27$, $p = .003$). These data have therefore also been transformed.

There was a significant Test*Drug interaction when responses in the first interval were analysed ($F_{1.8,48.2} = 4.07$, $p = .023$). Responses varied between the test sessions ($F_{1.8,48.2} = 4.87$, $p = .011$) but were not affected by drug treatment at reactivation ($F_{1,27} = 0.78$, $p = .385$). Subsequent analysis of this effect revealed that whilst animals treated with MK-801 varied levels of responding between the different sessions ($F_{2,28} = 7.65$, $p = .002$), animals treated with vehicle responded similarly in the baseline, test I and test II sessions ($F_{2,26} = 0.29$, $p = .753$). Post-hoc tests revealed that animals treated with MK-801 reduced their response levels in the first interval in comparison to their baseline responding in the first test ($p = .002$), but not the second ($p = .867$), such that responding

increased from the first test to the second test ($p = .013$; Figure 6.3B). In contrast, responding in the second interval did not vary between the test sessions (Session: $F_{2,54} = 1.19$, $p = .314$) and this did not vary between drug treatment groups (Session*Drug: $F_{2,54} = 1.63$, $p = .205$). Drug treatment did not affect overall levels of responding in the second interval ($F_{1,27} = 1.00$, $p = .327$). Response levels did not vary between sessions in either drug treatment group in the second interval (VEH: $F_{2,26} = 1.48$, $p = .247$; MK-801: $F_{2,28} = 1.32$, $p = .283$; Figure 6.3C). Raw response rates from all baseline and test sessions can be seen in Table 6.2.

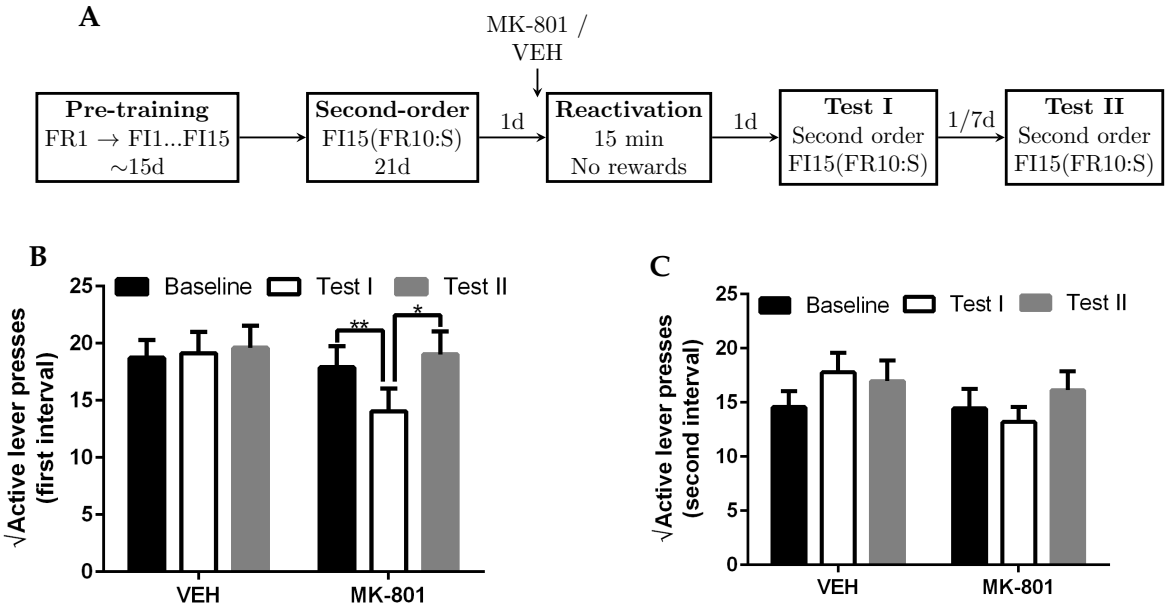


Figure 6.3: MK-801 administered before memory reactivation results in a subsequent short-lived decrease in well-established food-seeking behaviour **A**: Schematic of experimental procedures in Experiment 2. **B**: Active lever presses made in the first interval of the final session of second order and subsequent test sessions. **C**: Active lever presses made in the second interval of the final session of second order and subsequent test sessions. Data are represented as means +SEM. N=14/15 for each treatment group. * p<.05, ** p<.01

Interval Group	Baseline	Test I	Test II
1			
Vehicle	382.2 ± 56.80	411.7 ± 81.12	432.5 ± 77.54
MK-801	367.4 ± 71.21	253.7 ± 69.62	418.6 ± 84.04
2			
Vehicle	240.9 ± 54.56	358.9 ± 68.70	334.8 ± 64.88
MK-801	253.2 ± 61.57	201.0 ± 40.01	302.7 ± 56.24

Table 6.2: Raw response rates from the test session of Experiment 2. Data are represented as the mean number of active lever presses made in the respective interval $\pm$ SEM to 2 decimal points.

The number of inactive lever presses did not vary between sessions as a function of drug treatment and this was equally true in both intervals. In the first interval these effects were substantiated by a non-significant effect of Day ($F_{2,54} = 0.62$, $p = .544$), no Day*Drug interaction ($F_{2,54} = 0.81$, $p = .449$) and no overall effect of Drug ($F_{1,27} = 0.05$, $p = .832$; Table 6.3). A similar pattern of results was revealed when the number of lever presses made in the second interval was analysed (Day: $F_{1.8,47.5} = 0.98$, $p = .382$; Day*Drug: $F_{1.8,47.5} = 0.95$, $p = .393$; Drug: $F_{1,27} = 0.45$, $p = .506$; Table 6.3). The raw inactive lever press data for both intervals of the test session is presented in Table 6.4.

Interval Group	Baseline	Test	Test II
1			
Vehicle	3.1 ± 0.60	2.6 ± 0.49	2.3 ± 0.35
MK-801	2.6 ± 0.44	2.5 ± 0.33	2.6 ± 0.51
2			
Vehicle	3.0 ± 0.33	2.8 ± 0.44	2.0 ± 0.29
MK-801	2.9 ± 0.31	3.0 ± 0.77	2.9 ± 0.48

Table 6.3: Transformed inactive lever press data from the test sessions of Experiment 2. Data are presented as means ± SEM to 2 decimal points.

Interval Group	Baseline	Test	Test II
1			
Vehicle	14.0 ± 5.10	9.9 ± 4.54	7.1 ± 1.82
MK-801	9.3 ± 2.80	7.6 ± 1.75	10.6 ± 3.35
2			
Vehicle	10.6 ± 2.27	10.4 ± 3.44	4.9 ± 1.32
MK-801	9.7 ± 2.10	17.1 ± 9.74	11.7 ± 3.31

Table 6.4: Raw inactive lever press data from the test sessions of Experiment 2. Data are presented as means ± SEM to 2 decimal points.

Training and reactivation

All prospective groups showed similar levels of responding in the pre-training sessions. Data from these sessions were transformed since the data from several of the days' training were not normally distributed for rates of both active ($D_{15} > 0.22$, $p < .048$) and inactive ($D_{15} > 0.24$, $p < .024$) lever pressing. The day of training affected both the rate of active ($F_{4.5,99.5} = 42.44$, $p < .001$) and inactive lever pressing ($F_{3.1,82.4} = 19.52$, $p < .001$), with drug treatment having no effect on the pattern of acquisition (active: Day*Drug: $F_{4.5,99.5} = 0.78$, $p = .526$, inactive: Day*Drug: $F_{3.1,82.4} = 0.64$, $p = .767$)

or overall response levels on either lever (active: $F_{1,27}=0.01$, $p=.918$, inactive: $F_{1,27}=0.53$, $p=.473$; Figures 6.4A and 6.4B).

All groups responded similarly during the second-order sessions, regardless of the treatment that they would go on to receive at reactivation. Data from active lever pressing in the first and second interval, alongside the inactive lever pressing data in these intervals were not normal, and therefore all second-order training session data has been transformed ($D_{14}>0.23$, $p<.049$).

The number of responses made on the active lever varied throughout second-order training; the number of responses varied as a function of day of training in the first ($F_{6.0,161.0}=6.41$, $p<.001$) and second intervals ($F_{7.2,194.8}=5.90$, $p<.001$). This occurred similarly between prospective treatment groups in both intervals (Day*Drug, first interval: $F_{6.0,161.0}=1.48$, $p=.190$; second interval: $F_{7.5,201.3}=0.92$, $p=.565$). The overall number of active responses were also similar between prospective treatment groups in the first ($F_{1,27}<0.01$, $p>.999$; Figure 6.4C) and second intervals ($F_{1,27}=0.28$, $p=.599$; Figure 6.4D) of these training sessions.

The number of inactive lever presses made in the first interval of second-order training did not vary between sessions ($F_{7.5,201.3}=1.60$, $p=.131$), whilst it did for the second interval ($F_{8.7,234.6}=2.09$, $p=.033$). This was similarly true in both groups of animals (Day*Drug interaction, first interval: $F_{7.5,201.3}=0.92$, $p=.565$; second interval: Day*Drug: $F_{8.7,234.6}=0.87$, $p=.549$). The overall level of responding on the inactive lever was also similar between the two prospective drug treatment groups in both the first (Drug: $F_{1,27}=0.28$, $p=.599$; Figure 6.4E) and following interval (Day*Drug: $F_{8.7,234.6}=0.87$, $p=.549$; Figure 6.4F).

Prior drug treatment did not affect the number of responses made on either lever during the reactivation session. Responses on the active lever were not normally distributed for animals treated with MK-801 ($D_{15}=0.24$, $p=.024$) and inactive lever pressing data from neither group was normal ($D_{15}>0.26$, $p<.006$). Both groups made similar numbers of active ($F_{1,27}=1.85$, $p=.185$; Figure 6.4G) and inactive ($F_{1,27}=1.69$, $p=.204$; Figure 6.4H) lever presses during the reactivation session.

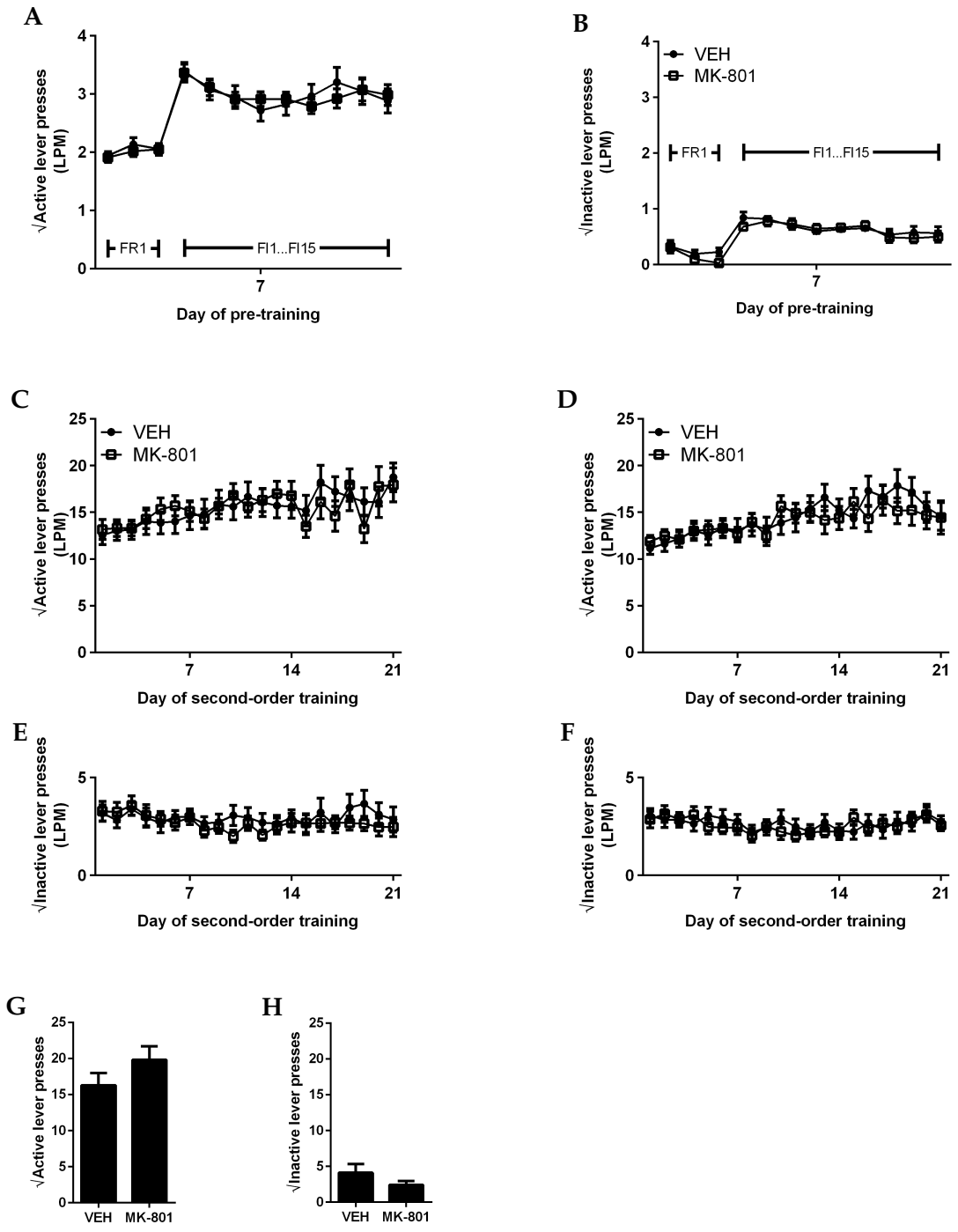


Figure 6.4: Pre-training, second-order training and reactivation data for Experiment 2. **A**: Rate of active lever pressing during pre-training sessions. **B**: Rate of inactive lever pressing during pre-training sessions. **C**: Number of active lever presses made in the first interval during second-order training sessions. **D**: Number of active lever presses made in the second interval during second-order training sessions. **E**: Number of inactive lever presses made in the first interval during second-order training sessions. **F**: Number of inactive lever presses made in the second interval during second-order training sessions. **G**: Active lever presses made in the reactivation session. **H**: Inactive lever presses made in the reactivation session. Data are represented as means +/±SEM. N=14/15 for each treatment group.

Experiment 3: NMDA receptor antagonism during multiple retrieval sessions of a well-established food-seeking memory has no effect on subsequent responding

Having demonstrated that MK-801 treatment prior to a single reactivation session resulted in a short-lived decrease in food-seeking behaviour, Experiment 3 aimed to produce a more robust, long-lasting deficit in responding in the second-order task. In an attempt to do this animals underwent a total of 3 reactivation sessions, each preceded with treatment with MK-801 or its vehicle (see Figure 6.5A).

One rat was excluded from this experiment after developing a tumour during training.

Test results

Data for all the baseline and test sessions met the assumption of normality for all treatment groups ($D_{12} < 0.24$, $p > .062$) and the data were analysed in their raw form.

Treatment with MK-801 prior to multiple memory reactivation sessions had no effect on subsequent food-seeking, with MK-801 and vehicle-treated rats showing similar patterns of responding in the first (Figure 6.5B) and second (Figure 6.5C) intervals. This was indicated by similar levels of responding in the first interval of the baseline and test sessions ($F_{2,42} = 0.91$, $p = .411$) regardless of treatment group (Session*Drug: $F_{2,42} = 1.23$, $p = .304$). The overall number of responses made in the second interval of the baseline and test sessions was also not affected by the drug administered prior to the memory reactivation sessions ($F_{1,21} = 0.15$, $p = .699$).

Analysis of the inactive lever press data from the test sessions revealed that these responses did not vary between days, as a function of drug treatment and this was equally true of both drug groups in both intervals. In the first interval these effects were indicated by a non-significant effect of Day ($F_{2,42} = 1.30$, $p = .284$), an absence of a significant Day*Drug interaction ($F_{2,42} = 0.78$, $p = .463$) and no main effect of Drug ($F_{1,21} = 0.40$, $p = .535$; Table 6.5). A similar pattern of results was revealed when responses made in the second interval were analysed (Day: $F_{1.3,26.6} = 2.80$, $p = .098$; Day*Drug: $F_{1.3,26.6} = 0.65$, $p = .463$; Drug: $F_{1,21} = 0.11$, $p = .743$; Table 6.5).

Power analysis

Whilst the effect of MK-801 treatment to reduce lever pressing in comparison to baseline at Test II was not significant it is apparent there was a numerical trend in the data resembling that of

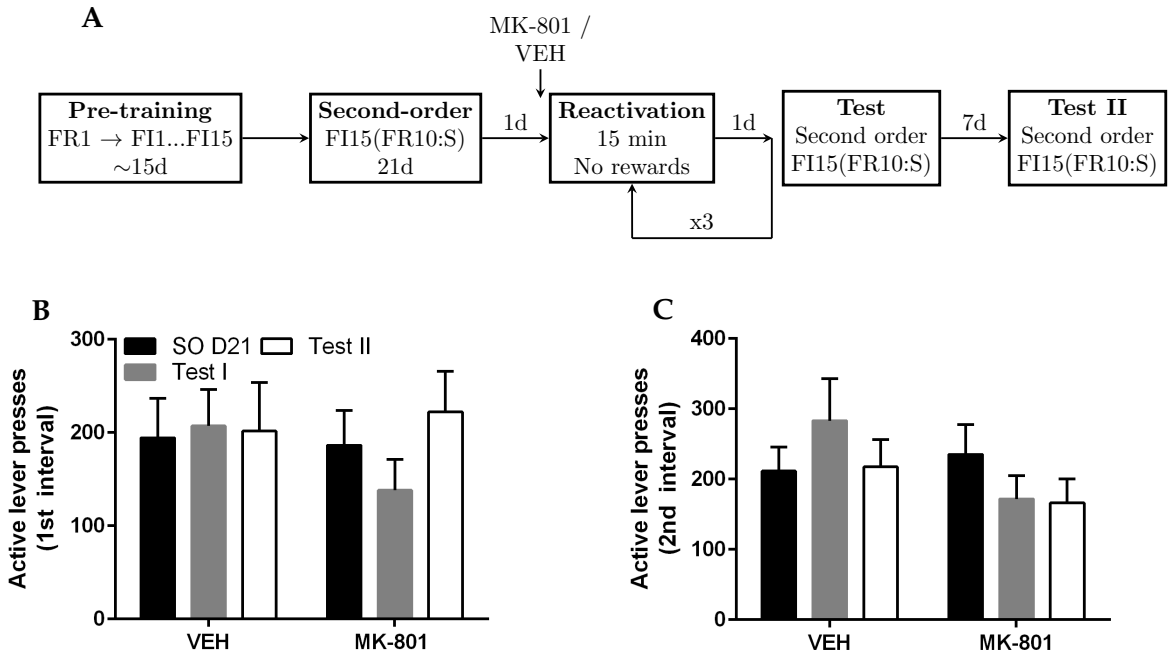


Figure 6.5: MK-801 administered before multiple reactivation sessions does not affect well-established food-seeking seeking **A**: Schematic of experimental procedures in Experiment 3, where the reactivation, and preceding drug treatment, were conducted 3 times. **B**: Active lever presses made in the first interval of the final session of second order and subsequent test sessions. **C**: Active lever presses made in the second interval of the final session of second order and subsequent test sessions. Data are represented as means +SEM. N= 11/12 for both treatment groups.

Interval Group	Baseline	Test	Test II
1			
Vehicle	10.4 ± 1.93	6.7 ± 1.33	7.3 ± 0.91
MK-801	7.0 ± 2.27	6.2 ± 2.35	7.2 ± 1.92
2			
Vehicle	8.5 ± 3.50	4.2 ± 0.87	5.0 ± 0.94
MK-801	6.5 ± 1.93	5.3 ± 1.72	3.9 ± 0.88

Table 6.5: Raw inactive lever press data from the test sessions of Experiment 3. Data are presented as means $\pm$ SEM to 2 decimal points.

Experiment 2. Power analysis was conducted in order investigate whether the smaller number of subjects in this experiment precluded the detection of a significant result. With the given effect size obtained when baseline and Test I were compared (d = 0.34), 70 rats would be required in order to achieve power of 0.8 with a p value of .05. Given that is much greater than the n of 14/15 that was used in Experiment 2 (for comparison, in this experiment the effect size was 1.11 and η^2 was 0.93) it

appeared that the failure to detect an amnestic effect of MK-801 treatment was not solely the result of the smaller number of animals used in this experiment [1].

Training and reactivation

Prospective groupings did not affect acquisition of the operant response in the sessions prior to the second-order schedule of reinforcement being introduced on either lever (Figures 6.6A and 6.6B). The data from these pre-training sessions violated assumptions of normality for both rates of active ($D_{11} > 0.26$, $p < .042$) and inactive lever pressing ($D_{12} > 0.26$, $p < .043$) so they have been transformed. Rates of active lever pressing varied across training sessions ($F_{1.9,40.5} = 69.97$, $p < .001$) similarly between prospective treatment groups (Session*Drug: $F_{1.9,40.5} = 1.74$, $p = .190$), with both groups responding at approximately similar rates across training sessions ($F_{1,21} = 0.06$, $p = .807$). The same was true of inactive lever presses: these varied between sessions ($F_{4.1,86.0} = 29.27$, $p < .001$) similarly across treatment groups (Day*Drug: $F_{4.1,86.0} = 1.84$, $p = .127$), with the overall rate of responding being similar between treatment groups ($F_{1,21} = 0.84$, $p = .369$).

Animals that would go on to receive treatment with vehicle or MK-801 responded at similar rates in the second-order training sessions on both the active (Figure 6.6C and 6.6D) and inactive (Figure 6.6E and 6.6F) lever. Data from several training sessions for both levers and intervals were not normally distributed for both treatment groups ($D_{11} > 0.25$, $p < 0.049$) so all the data from the second-order training sessions have been transformed. The number of active lever presses made in the first ($F_{5.8,115.1} = 4.11$, $p = .001$) and second ($F_{7.1,148.7} = 3.92$, $p = .001$) interval varied between training sessions, but this pattern was similar in both prospective treatment groups in both intervals (Day*Drug, first interval: $F_{5.8,115.1} = 0.67$, $p = .853$; second interval: $F_{7.1,148.7} = 0.69$, $p = .682$). The overall rate of responding in these sessions was similar treatment groups in both the first ($F_{1,21} = 0.14$, $p = .713$; Figure 6.6C) and following interval ($F_{1,21} = 0.15$, $p = .702$; Figure 6.6D).

ANOVAs similarly verified the lack of group differences in the level of inactive lever pressing throughout second-order training sessions. The day of training significantly affected the number of responses in both the first ($F_{6.2,131.0} = 3.30$, $p = .004$) but not the second interval ($F_{8.8,185.0} = 1.60$, $p = .121$). This was equally true in both prospective treatment groups in both intervals (Day*Drug, first interval: $F_{6.2,131.0} = 1.01$, $p = .450$; second interval: $F_{8.8,185.0} = 0.92$, $p = .511$). The treatment

[1] It could be argued that the square root transformation, that was necessary owing to the non-normally distributed data set in Experiment 2, reduced the error in this experiment, increasing the power. However, it was not appropriate to transform the data from Experiment 3 in order to compare the two experiments, not only because the raw data were normally transformed, but also because transformation of the data caused them to be become non-normal.

that animals would ultimately receive prior to the reactivation session did not affect the number of inactive lever presses made in the first ($F_{1,21} = 1.53$, $p = .230$; Figure 6.6E) or second ($F_{1,21} = 0.51$, $p = .483$; Figure 6.6F) interval.

The number of active lever presses did not differ between each of the reactivation sessions in either treatment group, although animals treated with MK-801 did exhibit slightly higher levels of responding in these sessions (Figure 6.6G). Active lever pressing data from the final reactivation session for animals treated with MK-801 were not normally distributed ($D_{12} = 0.25$, $p = .043$), so the data were transformed before statistical analysis took place. Active lever pressing did not vary between each of the reactivation sessions ($F_{2,42} = 2.07$, $p = .139$) and this was equally true between MK-801 and vehicle treated rats (Day*Drug: $F_{2,42} = 1.52$, $p = .230$). However, animals administered with MK-801 did respond more in the reactivation sessions than those treated with vehicle (Drug: $F_{1,21} = 5.20$, $p = .033$; Figure 6.6G). This is in accord with previous research demonstrating this dose of MK-801 can result in increased locomotor activity (Frantz and Hartesveldt, 1999).

Inactive lever presses from the reactivation sessions were analysed in a similar way and there was evidence of a slight increase in these responses between sessions in animals treated with vehicle, but not MK-801 (Figure 6.6H). Inactive lever pressing data from the vehicle-treated rats were not normally distributed for the first reactivation session ($D_{11} = 0.26$, $p = .040$) and for all reactivation sessions in MK-801-treated rats ($D_{12} > 0.30$, $p < .003$) so the data from these sessions have been transformed. Whilst the number of inactive lever presses made in the reactivation sessions did not appear to vary between sessions ($F_{2,42} = 1.77$, $p = .183$) this effect varied depending on pre-reactivation drug administration (Day*Drug $F_{2,42} = 3.90$, $p = .028$). Subsequent analysis revealed a significant effect of day in MK-801 ($F_{2,22} = 4.62$, $p = .021$) but not vehicle ($F_{2,20} = 0.27$, $p = .763$) treated rats. Post-hoc analysis of this effect in the MK-801 treated group revealed a non-significant increase in responding between the first and third reactivation session ($p = .054$). Pre-treatment with MK-801 did not affect the overall number of responses in these sessions ($F_{1,21} = 1.27$, $p = .272$; Figure 6.6H).

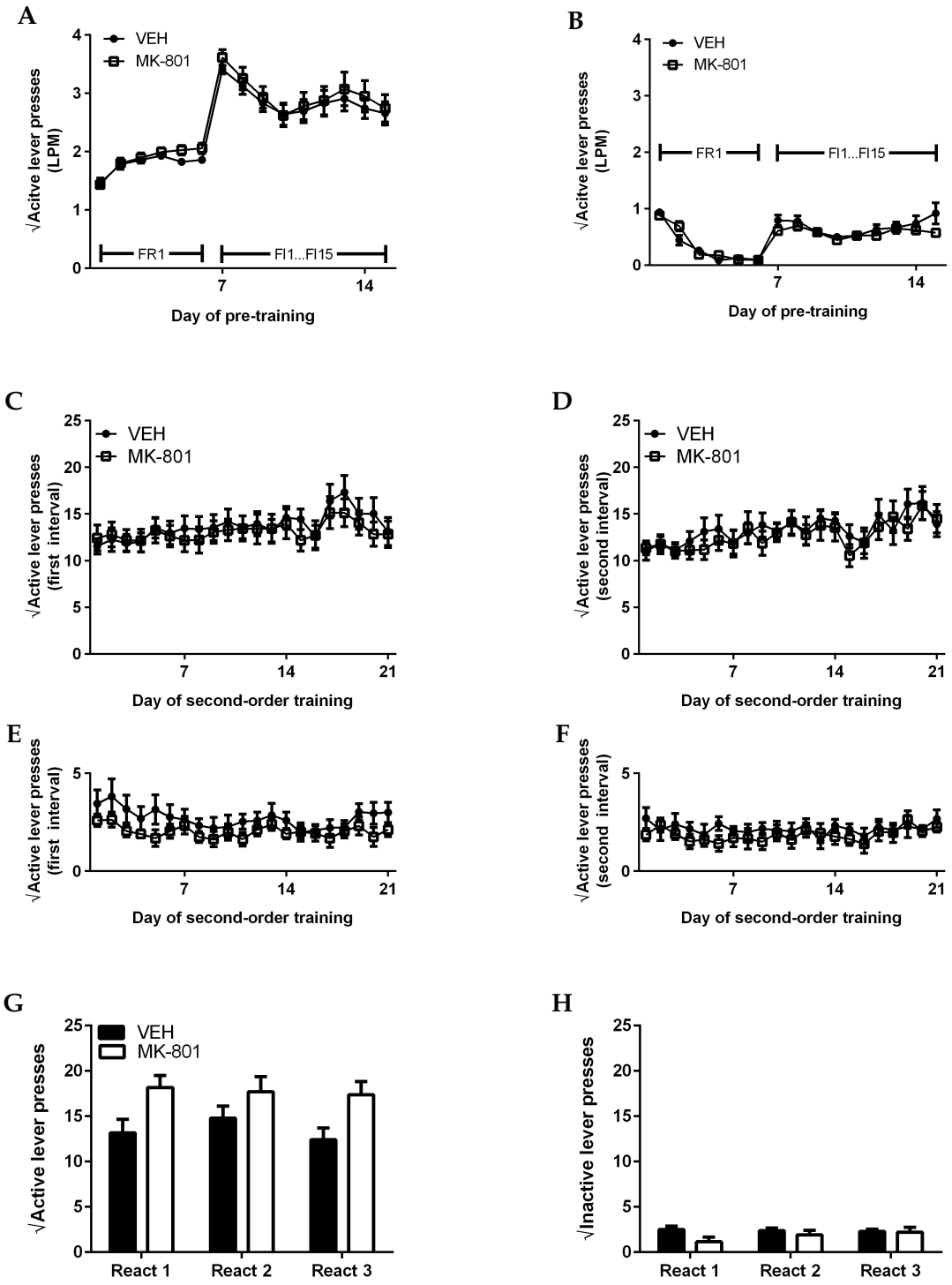


Figure 6.6: Pre-training, second-order training and reactivation data for Experiment 3. **A**: Rate of active lever pressing during pre-training sessions. **B**: Rate of inactive lever pressing during pre-training sessions. **C**: Number of active lever presses made in the first interval during second-order training sessions. **D**: Number of active lever presses made in the second interval during second-order training sessions. **E**: Number of inactive lever presses made in the first interval during second-order training sessions. **F**: Number of inactive lever presses made in the second interval during second-order training sessions. **G**: Active lever presses made in the reactivation sessions. **H**: Inactive lever presses made in the reactivation sessions. Data are represented as means +/±SEM. N=11/12 for each treatment group.

Discussion

Summary of results

Here it is reported that antagonism of NMDA receptors prior to reactivation of well-trained cocaine-seeking memory has no effect on subsequent drug seeking behaviour. By contrast, the same treatment resulted in a reduction in palatable food-seeking the next day. However, this decrease was short-lived and high rates responding of were rapidly reacquired. Multiple reactivations in combination with further drug treatments were not able to ameliorate the transitory nature of this effect.

Relationship to previous work

Memories underlying cocaine-seeking have previously been shown to reconsolidate (e.g. Fuchs *et al.*, 2009; Lee *et al.*, 2005b; Lee *et al.*, 2006a), a process that depends upon the activation of NMDA receptors (Milton *et al.*, 2008a). This is the first investigation of whether memories that have undergone extensive training, likely resulting in the formation of a cocaine seeking habit, reconsolidate in a similar fashion. It was not possible to disrupt reconsolidation of this memory. It has previously been demonstrated that strong memories, by virtue of having undergone multiple pairings (Suzuki *et al.*, 2004; Wang *et al.*, 2009) or being paired with a particularly strong US (Kwak *et al.*, 2012) are more resistant to destabilisation. It is possible that it is for these reasons that no effect of reconsolidation blockade of a cocaine seeking memory was reported; this process was not taking place. However, in some cases memory strength does not appear to affect destabilisation. For example it has been reported that propranolol is equally effective at preventing reconsolidation, regardless of the number of pairings a pavlovian fear memory has undergone (Taherian *et al.*, 2014). Similarly, the naturally occurring compound Garcinia is able to disrupt reconsolidation after 12 or 24 days of cocaine self-administration (Monsey *et al.*, 2017). However, in this study animals self-administered drugs on an FR1 schedule which is unlikely to result in the recruitment of cocaine seeking habits (Murray *et al.*, 2012); a factor which may have prevented reconsolidation from taking place in Experiment 1.

Habitual response patterns are by no means exclusive to drug-seeking behaviours; the progression from goal-directed to habitual responding was first characterised in responses for food reinforcers (Adams, 1982) and it is only comparatively recently that drug-seeking responses have been shown to be habitual (Corbit *et al.*, 2012; Dickinson *et al.*, 2002; Miles *et al.*, 2003; Zapata *et al.*, 2010). A

second experiment investigated reconsolidation of responses trained in fashion for devliery of a palatable food reinforcer, chocolate pellets. Here, NMDA receptor antagonism before reactivation of such a memory resulted in a decrease in food-seeking, suggesting this treatment disrupted reconsolidation of this association. This was evidenced by a decrease in food-seeking responses made in the first interval of the first test session.

There are several interpretations of the ability to disrupt food, but not cocaine associated memories. As discussed above, both the extent of training and the strength of the US can contribute towards the ability of a retrieval session to result in memory reconsolidation (Kwak *et al.*, 2012; Suzuki *et al.*, 2004; Wang *et al.*, 2009). Given cocaine's suggested ability to hijack natural reward systems (Hyman, 2005) it is possible this drug resulted in the formation of a stronger memory than in the food experiments, resulting in a resistance to destabilisation. The procedure used to train food and cocaine seeking responses also differed in that animals in the food experiments received fewer overall pairings; second-order sessions were limited to 5 infusions of cocaine each day or 2 chocolate pellet deliveries. The number of pairings a memory has undergone has previously shown to affect the propensity of a memory to destabilise in a given reactivation session (Suzuki *et al.*, 2004; Wang *et al.*, 2009). The combination of the larger number of cocaine pairings, combined with the increased strength of cocaine as a US may have acted in tandem to result in a stronger memory in animals trained to respond for cocaine, preventing the memory retrieval session from triggering reconsolidation in these animals.

It is perhaps worth noting that the response rates for animals seeking cocaine were considerably lower than those responding for food. This does not rule out the possibility that the memory underlying drug-seeking was stronger, since response rates as a measure of memory strength are confounded by potential differences in motivation. However, the lower response rates of animals self-administering cocaine may have precluded the detection of a reconsolidation deficit.

Whilst it did appear possible to disrupt reconsolidation of well trained food-seeking memory, the resultant deficit was apparently short-lived; responses in the subsequent interval, once both the CS and lever press were once again reinforced, were not affected by drug treatment at memory reactivation. The acute effects of μ-opioid antagonism on cocaine seeking (Giuliano *et al.*, 2013) or atomoxetine on heroin-seeking (Economidou *et al.*, 2011) result in similar interval-dependent effects and may suggest a reduction in the reinforcing effects of the drug cue, but not the ability of reinforcer delivery to affect responding, with the latter effect not necessarily related to the rewarding properties of the reinforcer delivered (Everitt and Robbins, 2000). However, it did not appear

that this was the result of an effect that was selective to the first interval, since responses in this period of the second test were also unaffected by drug treatment. This instead suggested that reminder of the associations underlying the seeking behaviour led to a loss of the apparent effects of reconsolidation blockade; an effect that might suggest that the initial deficit observed was the result of a retrieval, rather than a storage deficit (Miller and Springer, 1973).

It has been previously reported that disruptions of memory reconsolidation resulting in decreased responding in second-order schedules of reinforcement are long-lasting and persist following reminder of the CS-US pairing (Lee *et al.*, 2006a). Deficits arising from disruptions of discrete cue fear memory reconsolidation do not undergo spontaneous recovery (Duvarci and Nader, 2004). In contrast, the effects of disrupting reconsolidation of contextual fear associations are lost with the passage of time (Lattal and Abel, 2004) and are particularly sensitive to return with reminder shocks (Fischer *et al.*, 2004; Trent *et al.*, 2015). The distinction in the results of contextual and cued memory reconsolidation disruptions may reflect that whilst some aspects of the memory trace are lost, others are maintained. A contextual fear association is likely comprised of (at least) two components: one of the configural representation of the context (likely hippocampal) and another pairing this with shock (likely dependent on the BLA, see Hall *et al.*, 2001a). Disruptions of protein synthesis within the hippocampus (as in Fischer *et al.*, 2004; Lattal and Abel, 2004; Trent *et al.*, 2015) may disrupt the contextual representation, but leave its pairing with shock intact. This latter association that remains is sufficient to support return of fear following reminder or the passage of time. The results of Experiment 2 may be explained by similar mechanisms; one aspect of the memory was reconsolidated, resulting in a decrease in responding in the first interval, but following reinforcer delivery other components of the memory trace (that were not reactivated) that underlie responding were able to once again take control of food-seeking.

It is possible that the decreased responding in animals treated with MK-801 occurred as a result of state-dependency. It has been shown that retrieval deficits occurring as a result of central and systemic post-reactivation protein synthesis inhibition can be reversed with pre-test administration of the same drug (Gisquet-Verrier and Riccio, 2012). Furthermore, treatment with lithium chloride (LiCl), which results in gastric malaise and does not affect protein synthesis (Squire *et al.*, 1975), can lead to similar effects (Gisquet-Verrier *et al.*, 2015). Treatment with MK-801 before a test session can also reverse apparent amnestic effects occurring as a result of the prior administration of this drug (Flint Jr. *et al.*, 2013). One explanation of these results is that during reactivation the internal state of the animal (i.e. whether there is a drug on board) becomes integrated into the memory trace, such that it can be only retrieved following administration of the same compound (Gisquet-Verrier

and Riccio, 2012). It is possible that treatment with MK-801 in Experiment 2 resulted in a similar retrieval deficit. Delivery of the reinforcer during the test may have unleashed it from its state-dependent state, permitting retrieval in MK-801 treated animals in subsequent intervals. Testing this hypothesis, however, is problematic since administration of MK-801 can result in hyperactivity and increased locomotion (Frantz and Hartesveldt, 1999), confounding conclusions made from lever press data occurring as a result of the acute effects of this treatment.

Another possibility was that the food-seeking memory was not fully able to destabilise in a single reactivation session; previous demonstrations have suggested that strong memories may be more amenable to reconsolidation blockade following multiple reactivation sessions (Robinson and Franklin, 2010). A third experiment addressed this possibility; animals underwent three reactivation sessions, each preceded by drug (or vehicle) treatment. However, this did not appear to result in a long-lasting deficit in food-seeking; instead the initial effect of MK-801 administration to reduce this behaviour was lost, with both treatment groups responding similarly at test. It is known that the extent of retrieval is a critical determinant in exposing a memory to disruption with amnestic agents. As reactivation sessions come to deviate from acquisition more and more the window for reconsolidation to occur can close, resulting an insensitivity of the memory to disruption (Merlo *et al.*, 2014; see also Alfei *et al.*, 2015; Flavell and Lee, 2013; Merlo *et al.*, in preparation; Reichelt and Lee, 2013a). It is possible that the loss of the amnestic effect of MK-801 treatment was the result of the recruitment of a similar 'limbo' process.

It is perhaps worth noting that the lack of an amnestic effect of multiple treatments with MK-801 makes a state-dependent account of Experiment 2 unlikely. If MK-801 treatment had led to the reactivated memory becoming state-dependent it would be expected that three treatments with MK-801 would be more likely result in such an effect, yet this did not affect responding at test. It was also possible the results of Experiment 2 were caused by effects of MK-801 treatment that were unrelated to the fact it was administered prior to the memory reactivation session. However, the fact that this effect was not observed when the same drug was given 3 times suggested that this was not the case, although the possibility cannot be excluded.

Implications for subsequent research

For the first time in this thesis NMDA receptor antagonism resulted in a decrease in subsequent responding. However, this effect was only found when animals were trained to respond for a food

reinforcer, with no effect being reported using similar conditions for cocaine associated memories. Furthermore, this deficit was short-lasting, exposing the possibility that it was not due to a reconsolidation deficit, since this should lead to loss of the memory, preventing its rapid recovery. With these apparent shortcomings of using NMDA receptor antagonism to prevent reconsolidation, alongside the frequent inability to prevent this process in previous chapters, subsequent investigations attempted to characterise the deficits in memory expression occurring as a result of this treatment. The behavioural conditions that permit a retrieval session to lead to the exposure of the reactivated memory to interference with amnestic agents were also explored.

Chapter 7: An analysis of the neurochemical and behavioural requirements of fear memory reconsolidation

Introduction

Previous attempts to prevent reconsolidation in Chapters 4 and 5 were unable to replicate previous studies showing the dependence of this process on N-methyl-D-aspartate (NMDA) receptor activation. Whilst antagonism of these receptors did result in a decrease in food-seeking in Chapter 6 this effect was short-lived and did not appear to reflect an entirely amnestic effect of this treatment. Experiments in this chapter attempted to prevent fear memory reconsolidation, manipulating the behavioural parameters of the reactivation and training sessions used, alongside the use of different pharmacological compounds and timing of their administration. These experiments were conducted with the view that these results may also shed light on the requirements for destabilisation and reconsolidation of appetitive associations.

The processes underlying reconsolidation of appetitive and aversive memories appear to be similar; reactivation of both of these memories requires prediction error (PE) (Díaz-Mataix *et al.*, 2013; Reichelt and Lee, 2013c), with associations undergoing extended training requiring longer reactivation sessions (and thus more extensive PE) to destabilise (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014; Reichelt and Lee, 2013a; Suzuki *et al.*, 2004). The reconsolidation of both of these associations also requires NMDA (e.g. Lee and Everitt, 2008a; Lee *et al.*, 2006b; Merlo *et al.*, 2014; Milton *et al.*, 2008a; Wouda *et al.*, 2010) and β-adrenergic (Dębiec and Ledoux, 2004; Milton *et al.*, 2008b) receptor activation, alongside protein synthesis (e.g. Dunbar and Taylor, 2016; Merlo *et al.*, 2015; Nader *et al.*, 2000).

The training protocols used in fear memory studies allow for close control of every parameter resulting in the formation of these associations, unlike the instrumental protocols used in the previous chapters, where acquisition was contingent on an animal's behaviour. This allows for parametric manipulation of the strength of the memory and this is less likely to be affected by individual

differences. A better understanding of the behavioural and neurochemical characteristics of reconsolidation of fear memories may provide an insight into the reasons for frequently not being able to prevent reconsolidation of reward-related memories in previous chapters. This information may in turn be of benefit in maximising the likelihood of blocking this process for maladaptive memories underlying psychological conditions such as post-traumatic stress disorder (PTSD) and drug addiction.

In line with many other experiments of this thesis it was not possible to replicate previous demonstrations of the blockade of fear memory reconsolidation with NMDA receptor antagonism (Lee *et al.*, 2006b; Merlo *et al.*, 2014). Further experiments in this chapter therefore aimed to investigate why this might be the case. Although there are reports of NMDA receptor antagonism preventing reconsolidation of fear memories (Lee *et al.*, 2006b; Merlo *et al.*, 2014; Milton *et al.*, 2008a), this treatment can also prevent destabilisation of these associations (Ben Mamou *et al.*, 2006; Yu *et al.*, 2016). It is possible that this latter effect meant that pre-reactivation MK-801 administration prevented destabilisation, meaning it was not possible to prevent reconsolidation, as the memory had not been able to destabilise. In order to avoid these effects one possibility is to conduct the reactivation session in a drug-free state and administer the NMDA receptor antagonist immediately after the session, when destabilisation should have taken place and the memory is being reconsolidated. In the following experiments animals were treated with MK-801 either before or after the memory reactivation session and the effects on subsequent fear expression explored.

It is also possible that NMDA receptor activation has a fleeting role in reconsolidation and may only be required for this process during retrieval. Immediately after reactivation (in the order of seconds) the protein synthesis cascades required for reconsolidation to take place may have already been activated. It is known that NMDA receptor activation is required for the upregulation of *zif-268* and extracellular signal-regulated kinase (ERK) that occurs in when reward-related and aversive memories reconsolidate, respectively (Merlo *et al.*, in preparation; Milton *et al.*, 2008a). This could mean that once the memory has been reactivated, and these cellular cascades activated, NMDA receptor activation is no longer required for reconsolidation. Paradoxically, this would mean that MK-801 should be given before the session to prevent reconsolidation but its potential effect to block destabilisation makes this problematic. Alternatively, NMDA receptor antagonists should be given after the session, thus leaving destabilisation intact, but at this latter time point NMDA receptors may no longer be required for reconsolidation. Whilst destabilisation and reconsolidation do both rely on NMDA receptor activation, there is a double-disassociation in the receptor subtypes required for these processes to occur; destabilisation requires GluN2B receptor activation

and restabilisation GluN2A receptors (Ben Mamou *et al.*, 2006; Milton *et al.*, 2013). Attempts to prevent reconsolidation should therefore target GluN2A receptors. With this in mind the effect of pre-reactivation administration of the NMDA receptor antagonist CPP, which shows a preference for the GluN2A subunit (Feng *et al.*, 2004; Feng *et al.*, 2005), on subsequent fear expression was explored.

It is relatively well established that memories do not reconsolidate each time they are retrieved; this process only occurs when novel information is presented (e.g. Alfei *et al.*, 2015; Pedreira *et al.*, 2004; Sevenster *et al.*, 2013), a characteristic that likely relates to the function of reconsolidation to update existing memories (Lee, 2009; Nader and Einarsson, 2010). Although the memory reactivation sessions described in previous chapters were conducted in the absence of a reinforcer, and this is typically sufficient to engage reconsolidation mechanisms, it is possible that this alone was not sufficiently different from training to induce destabilisation. Novel contextual information increases the likelihood of reconsolidation of an object recognition memory taking place, despite no new information directly related to the object being presented (Winters *et al.*, 2009). Whilst reactivation of a cued fear memory in a novel context results in reconsolidation that is sensitive to protein synthesis inhibition, reactivation in a familiar context had no such effect (Jarome *et al.*, 2015). Furthermore, the majority of memory reactivation sessions that result in destabilisation and subsequent reconsolidation are conducted in a novel context (e.g. Ben Mamou *et al.*, 2006; Dębiec and Ledoux, 2004; Duvarci and Nader, 2004; Nader *et al.*, 2000), although there are reports of memory destabilisation occurring without a context shift (Lee *et al.*, 2006b; Merlo *et al.*, 2014). The possibility that the absence of novel contextual information at reactivation was preventing labilisation of the memory was therefore also investigated. Because of the aforementioned issues of pre-reactivation MK-801 preventing memory destabilisation the effects of administering MK-801 both before and after reactivation in a novel context were explored. The presence of novel contextual information did not appear to promote destabilisation.

Whilst it is known that novelty is required for reconsolidation to occur, stronger memories may require a proportionally larger violation of expectancies to destabilise. For example, longer reactivation sessions are required to trigger reconsolidation of contextual fear memories that have undergone extensive training (Suzuki *et al.*, 2004). The possibility that insufficient PE was preventing destabilisation taking place was therefore investigated with an increased number of conditioned stimulus (CS) presentations within the retrieval session. However, because longer reactivation sessions are not always effective in overcoming a resistance to destabilisation (Wang *et al.*, 2009) another experiment reduced the training to reactivation CS ratio with a weaker training protocol, reducing

the number of CS-unconditioned stimulus (US) pairings during memory acquisition, rather than at reactivation.

It was not clear whether MK-801 would be able to prevent reconsolidation, should it be taking place. In order to confirm that this drug can disrupt memory processes the ability of NMDA receptor antagonism to prevent fear memory *acquisition* was then investigated, with MK-801 administered prior to training. Experiments using animals that had been previously trained in an appetitive task revealed no effect of this manipulation. Because re-learning can occur via NMDA receptor-independent mechanisms (Bannerman *et al.*, 1995; Hardt *et al.*, 2009; Langton and Richardson, 2008; Langton and Richardson, 2010; Sanders and Fanselow, 2003; Saucier and Cain, 1995; Wiltgen *et al.*, 2010) the possibility that this pre-training was affecting the susceptibility of acquisition to antagonism of these receptors was investigated. The inability of NMDA receptor blockade to prevent consolidation was replicated using both naïve and animals pre-trained to fear a visual stimulus.

Since NMDA receptor antagonists were found to be ineffective oin preventing consolidation and reconsolidation the use of these drugs as amnestic agents was discontinued. Subsequent experiments inhibited synthesis of the kinase mammalian target of rapamycin (mTOR) following memory reactivation. The inhibitor of this pathway, rapamycin disrupts mTOR signalling (Hoeffer and Klann, 2010), known to be required for synaptic plasticity, and has previously been used to prevent fear memory reconsolidation (Blundell *et al.*, 2008; Gafford *et al.*, 2011; Hoffman *et al.*, 2015). Since this compound did not appear to prevent this process, with the resultant deficits apparently caused by state-dependent learning (Gisquet-Verrier and Riccio, 2012; Gisquet-Verrier *et al.*, 2015), subsequent experiments attempted to prevent reconsolidation with protein synthesis inhibition within the basolateral amygdala (BLA), a manipulation frequently used to prevent this process (e.g. Ben Mamou *et al.*, 2006; Jarome *et al.*, 2015; Merlo *et al.*, 2015; Milton *et al.*, 2013; Nader *et al.*, 2000).

Methods

Summary

Animals were trained to fear an auditory stimulus through presentations of the CS with a mild footshock. The CS was then presented in subsequent long-term memory test (LTMT) sessions either with the view of reactivating the memory (Experiments 1-7, 10 & 11) or investigating the consequences of pre-training administration of amnestic agents (Experiments 8 & 9).

Reactivation sessions were conducted after (Experiment 1) or immediately before (Experiment 2) treatment with the NMDA receptor antagonist MK-801. In Experiment 3 a different NMDA receptor antagonist, CPP, known to more selectively target GluN2A subunit-containing NMDA receptors, was given before the memory reactivation session. In Experiments 4 and 5 the reactivation session took place in a novel context, and the effects of pre (Experiment 4) and post-reactivation (Experiment 5) administration of MK-801 investigated. The number of CS presentations in the reactivation (Experiment 6) and training sessions (Experiment 7) was then altered and the susceptibility of the memory destabilisation following these manipulations explored with pre-reactivation MK-801 treatment. In Experiment 8 & 9 MK-801 was administered prior to the fear training session in animals that had been used in an appetitive experiment, those pre-trained to fear a visual CS and naïve animals. Experiments 10 and 11 used systemic rapamycin and intra-BLA anisomycin, respectively, in further attempts prevent memory reconsolidation.

Experiments that attempted to prevent reconsolidation are summarised in Table 7.1. Procedures were conducted as in General methods except where stated.

	Treatment		Novel context	CS-US	CSs at
Exp.	Drug	Timing	for react?	Pairings	reactivation
1	MK-801	Before	✗	2	1
2	MK-801	After	✗	2	1
3	CPP	Before	✗	2	1
4	MK-801	Before	✓	2	1
5	MK-801	After	✓	2	1
6	MK-801	Before	✗	2	2
7	MK-801	Before	✗	1	1
10	RAPA	After	✗	2	1
11	BLA-ANI	After	✗	2	1

Table 7.1: Summary of pharmacological and reactivation parameters manipulations used to attempt to prevent fear memory reconsolidation in Chapter 7. Not depicted are Experiments 8 & 9, where pre-training MK-801 was administered before training in an attempt to prevent fear memory consolidation, rather than reconsolidation. RAPA - rapamycin, ANI - anisomycin.

Subjects

Subjects were 170 Lister-Hooded rats weighing 260-460g before the start of experiments. In some cases animals had been previously used in studies using food as a reinforcer; where this is the case this is mentioned in the description of each experiment below.

Apparatus

Animals were trained in Paul Fray conditioning chambers. In experiments where the boxes were modified to form a novel context for the reactivation and test sessions the grid floors were covered with a black acrylic sheet, the normally clear Perspex door had striped wallpaper attached to it and light was provided by a light on the wall, rather than above the operant chamber (of the same wattage as the houselight and colour used in the training context). Pilot studies with context-induced renewal confirmed that animals were able to discriminate between these two contexts. Because the acrylic floors prevented contact with the shocking grid floors it was not possible to counterbalance which contexts served as the training and reactivation/test contexts.

Experiment 1: Antagonism of NMDA receptors with MK-801 before memory retrieval

Animals were first trained to fear an auditory CS through 2 pairings of this stimulus with a mild foot shock. The following day animals underwent a memory reactivation session, consisting of a single CS presentation in the absence of shock delivery. 30 minutes before this session animals were treated with either the NMDA receptor antagonist (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801) (Abcam) or its vehicle. 24h later the CS was presented in post-reactivation long-term memory test (PR-LTMT)1 and 7d later in PR-LTMT2. This experiment was a replication of previous experiments that have been conducted in the lab and have successfully demonstrated an involvement of NMDA receptors in memory reconsolidation, as indicated by a decreased level of fear expression in the subsequent memory tests (Lee *et al.*, 2006b; Merlo *et al.*, 2014).

Experiment 2: Antagonism of NMDA receptors with MK-801 after memory retrieval

This experiment was conducted as in Experiment 1, except that MK-801 was given immediately after, rather than before, the memory reactivation session. Animals were tested 24h after reactivation and not tested again.

Experiment 3: Antagonism of NMDA receptors with CPP before memory retrieval

The training and reactivation protocols were conducted as in Experiment 1, except that 60 minutes before the memory reactivation animals were administered with (±)-3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) or its vehicle and the memory tested 24h and 7d later.

Experiment 4: Antagonism of NMDA receptors with MK-801 before memory retrieval in a novel context

This experiment was conducted exactly as Experiment 1, with MK-801 being given before the reactivation session, except that the reactivation and subsequent test sessions occurred in a novel context.

Experiment 5: Antagonism of NMDA receptors with MK-801 after memory retrieval in a novel context

This experiment was conducted exactly as Experiment 4, except that MK-801 was given immediately after, rather than before, the memory reactivation sessions.

Experiment 6: Antagonism of NMDA receptors with MK-801 before a retrieval session consisting of 2-CS presentations

This experiment was conducted exactly as Experiment 1, except that the reactivation session consisted of 2 CS presentations, separated by 1 minute.

Experiment 7: Antagonism of NMDA receptors with MK-801 before retrieval of a weakly trained fear memory

This experiment was conducted as Experiment 1, except that animals underwent a single pairing between the CS and US. Animals in this experiment had been used in a prior appetitive experiment which included administration of MK-801 (Sigma) in half of the animals. Prior treatment groups were counterbalanced across drug groups in this experiment.

Experiment 8: Antagonism of NMDA receptors with MK-801 during fear memory acquisition

Animals were administered with MK-801 or its vehicle 5 minutes before the training session, which consisted of 2 CS-US pairings. The CS was presented in LTMT sessions 24h and 7d later. Animals in this experiment had been used in a prior appetitive experiment without drug administration.

Experiment 9: Antagonism of NMDA receptors with MK-801 prior to fear memory acquisition in naïve or pre-trained animals

After context habituation, but before auditory fear conditioning, half the animals were trained to fear a light stimulus. During this period the animals that were not undergoing pre-training remained in the operant chambers for the same duration as those that were, but without CS presentation or shock delivery. The following day animals underwent auditory CS fear training with half of the pre-trained and naïve animals receiving MK-801 or its vehicle before the sessions. Three days after auditory CS fear training all animals were presented with the light CS. Tests of freezing to the auditory CS presentations were conducted 24h and 8d after training sessions.

Experiment 10: Inhibition of the mTOR pathway following memory retrieval

Rapamycin or its vehicle was administered immediately following the reactivation session, conducted as in Experiment 1. Animals were tested the next day, and 7d after the first test. 3d after the 2nd PR-LTMT animals underwent a further test (all) having been administered with rapamycin 60 minutes before the test. Because rapamycin treatment resulted in a profound decrease in body weight all animals were given a soaked diet 6d after drug treatment in an attempt to curtail any adverse effects resulting from this weight loss.

Experiment 11: Effects of intra-BLA anisomycin following memory retrieval

Animals in Experiment 10 had bilateral cannulae implanted towards the BLA under ketamine and xylazine anaesthesia as described in General methods. Anisomycin or phosphate buffered saline (PBS) was infused immediately into the BLA following the memory reactivation session, conducted as in Experiment 1. The effects of these manipulations on memory expression were tested 24h and 8d after reactivation.

Statistical analysis

Data were analysed using mixed-design analyses of variance (ANOVAs). Freezing during the CS in the training, reactivation and test sessions was analysed as a repeated-measures factor (Session), with drug treatment at reactivation as a between-subjects factor (Drug). In Experiment 9 Pre-training was treated as a between-subjects factor. Freezing during the training sessions and

the one minute prior to the presentation of the CS was analysed in a similar fashion, in separate ANOVAs.

Results

Experiment 1: Effects of antagonism of NMDA receptors with MK-801 before memory retrieval

MK-801 was administered before the memory reactivation session (see Figure 7.1A), a treatment that has previously shown to result in a reduction in fear expression in a later tests (Lee *et al.*, 2006b; Merlo *et al.*, 2014).

MK-801 treatment had no effect on memory expression acutely, or the day after memory reactivation session. However, when tested 7 days later animals given MK-801 showed increased fear expression to the CS (Figure 7.1B). This was indicated by no overall effect of Drug ($F_{1,10} = 4.19$, $p = .068$) but a significant Drug*Test interaction ($F_{1.3,13.1} = 8.91$, $p = .007$). Pairwise comparison of this effect showed that animals treated with MK-801 exhibited increased freezing in response to the CS in comparison to vehicle treated controls at PR-LTMT2 (Figure 7.1B).

The results of the test sessions could not be accounted for by differences in levels of freezing during training (Table 7.4) or before the CS was presented in reactivation and test sessions (Table 7.5).

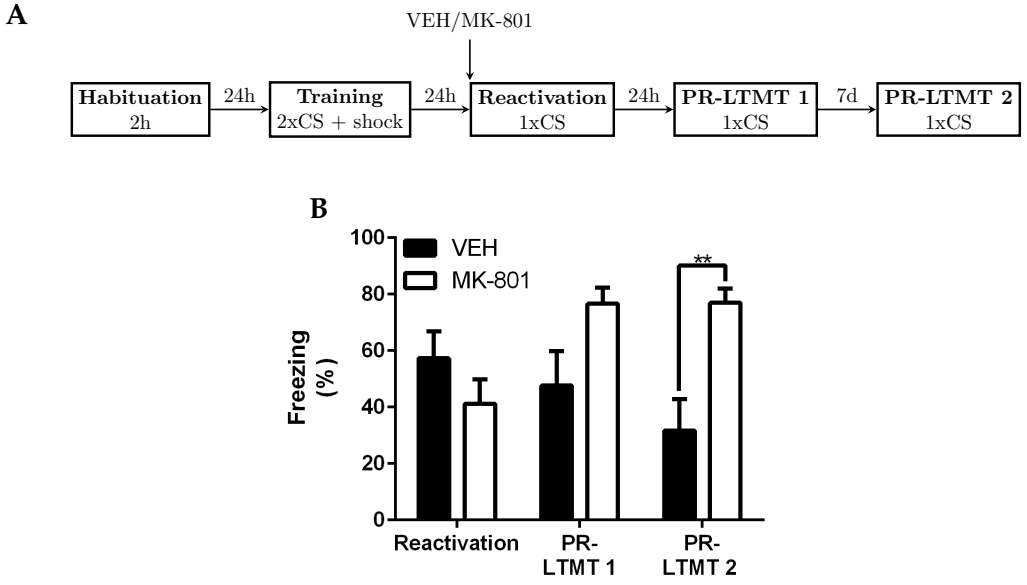


Figure 7.1: MK-801 administered before memory retrieval resulted in an enhancement of subsequent fear expression. **A**: Schematic of experimental procedures in Experiment 1. **B**: Freezing during the CS presentation in long-term memory tests. N=6 for both groups. Bars represent means +SEM. ** $p < .01$

Experiment 2: Effects of antagonism of NMDA receptors with MK-801 after memory retrieval

NMDA receptor antagonism before memory reactivation can prevent not only reconsolidation, but also destabilisation of an auditory fear memory (Ben Mamou *et al.*, 2006). This experiment antagonised NMDA receptors immediately after the session (see Figure 7.2A). This should mean destabilisation has already taken place and the memory is labile and MK-801 should only be able to prevent reconsolidation without affecting destabilisation.

Administration of MK-801 immediately after the reactivation session had no effect on subsequent freezing. This was indicated by no overall effect of Drug ($F_{1,10} = 0.03$, $p = .873$) and no Drug*Test interaction ($F_{1,10} = 0.97$, $p = .347$; Figure 7.2B).

There were no differences in freezing between groups in training (Table 7.4) or before the presentation of the CS in the reactivation and test sessions (Table 7.5).

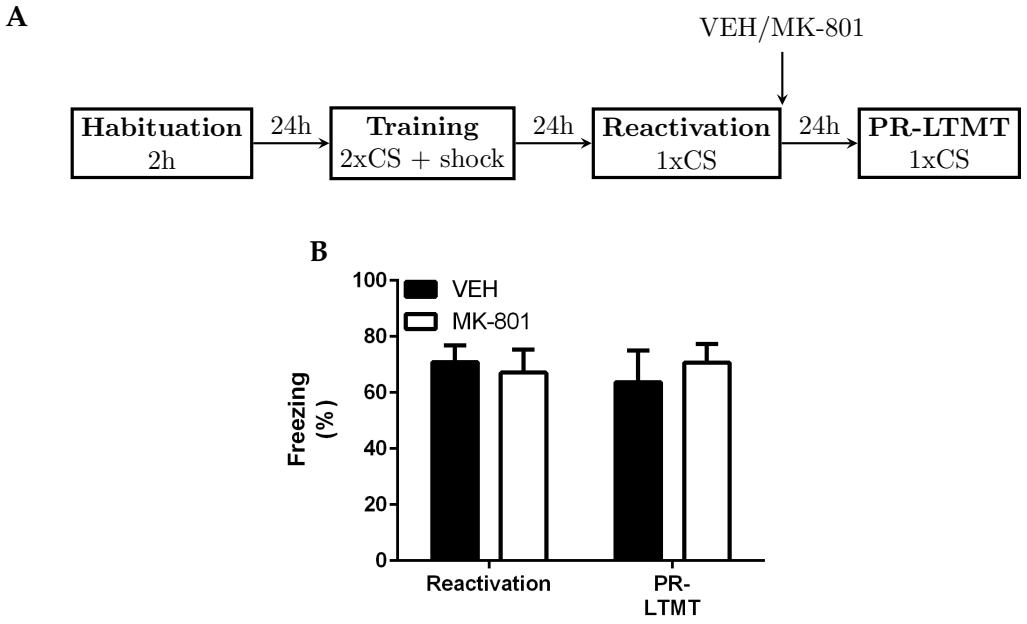


Figure 7.2: MK-801 administered after memory retrieval had no effects on fear expression. **A**: Schematic of experimental procedures in Experiment 2. **B**: Freezing during the CS presentation in long-term memory tests. N=6 for both groups. Bars represent means +SEM.

Experiment 3: Effects of antagonism of NMDA receptors with CPP before memory retrieval

In order to investigate the specificity of the memory enhancing effect of pre-reactivation MK-801 reported in Experiment 1, a different NMDA receptor antagonist, CPP, was used (see Figure 7.3A). This drug also has greater affinity for the GluN2A subunit of the NMDA receptor (Feng *et al.*, 2004;

Feng *et al.*, 2005), which has been suggested to underlie the amnestic effects of NMDA receptor antagonism at memory retrieval (Milton *et al.*, 2013).

Treatment with CPP had no effect on freezing levels in any of the memory tests, as indicated by no overall effect of Drug ($F_{1,10} = 0.45$, $p = .518$) and no Drug*Test interaction ($F_{2,20} = 1.88$, $p = .158$; Figure 7.3B).

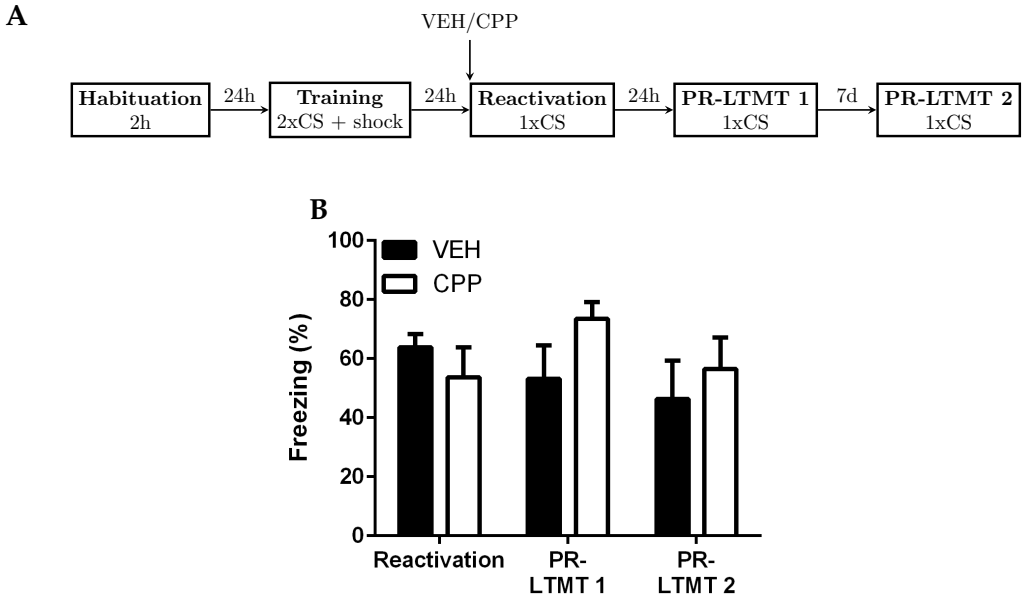


Figure 7.3: CPP administered before memory retrieval had no acute or long lasting effects on fear expression. **A**: Schematic of experimental procedures in Experiment 3. **B**: Freezing during the CS presentation in long-term memory tests. Bars represent means +SEM.

There were no differences between groups during training (Table 7.4) or during the pre-CS periods (Table 7.5).

Experiment 4: Effects of antagonism of NMDA receptors with MK-801 before memory retrieval in a novel context

One possibility arising from the previous experiments was that the reactivation session was not able to engage destabilisation and subsequent reconsolidation mechanisms. Given that previous research has suggested that novel contextual information may be required for reconsolidation to take place (Jarome *et al.*, 2015; Winters *et al.*, 2009), MK-801 was given before reactivation in a novel context (see Figure 7.4A).

Administration of MK-801 before the reactivation session resulted an acute decrease in freezing, but groups did not differ in memory expression in the PR-LTMTs (Figure 7.4B). This was supported

by a non-significant effect of Drug on freezing ($F_{1,10} = 2.41$, $p = .152$) but a significant Drug*Test interaction ($F_{2,20} = 24.72$, $p < .001$). Further analysis revealed a decrease in freezing during the reactivation session in animals treated with MK-801 but at no other time points (Figure 7.4B).

There were no differences between the prospective groups during training (Table 7.4). Freezing before the CS was presented in the PR-LTMTs, was, however lower in animals treated with MK-801 at reactivation (Table 7.5), potentially indicating that associations between the context and shock had been disrupted by NMDA receptor antagonism at reactivation.

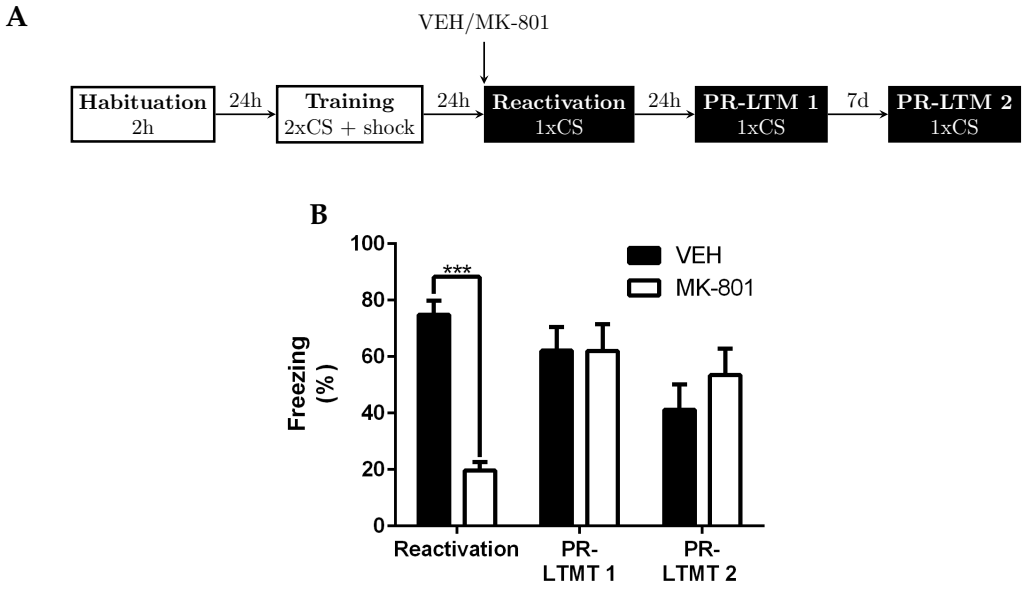


Figure 7.4: MK-801 administered before memory retrieval in a novel context resulted in an acute, but not long lasting, deficit in freezing. **A**: Schematic of experimental procedures in Experiment 4. **B**: Freezing during the CS presentation in long-term memory tests. N=6 for both groups. Bars represent means +SEM. ***$p < .001$

Experiment 5: Effects of antagonism of NMDA receptors with MK-801 after memory retrieval in a novel context

As mentioned previously, pre-reactivation NMDA receptor antagonism can prevent destabilisation (Ben Mamou *et al.*, 2006; Yu *et al.*, 2016). It remained possible that MK-801, when given before reactivation in a novel context, prevented destabilisation that would otherwise be occurring. In Experiment 5 MK-801 was administered directly after a reactivation session in a novel context (see Figure 7.5A).

MK-801 given immediately after the reactivation session had no effects on freezing in subsequent sessions, as indicated by no overall effect of Drug ($F_{1,10} = 2.27$, $p = .163$) and a non-significant Drug*Test interaction ($F_{1,20} = 0.16$, $p = .851$; Figure 7.5B).

There were no differences in freezing between the groups in training (Table 7.4) or the pre-CS periods of the reactivation and PR-LTMT sessions (Table 7.5).

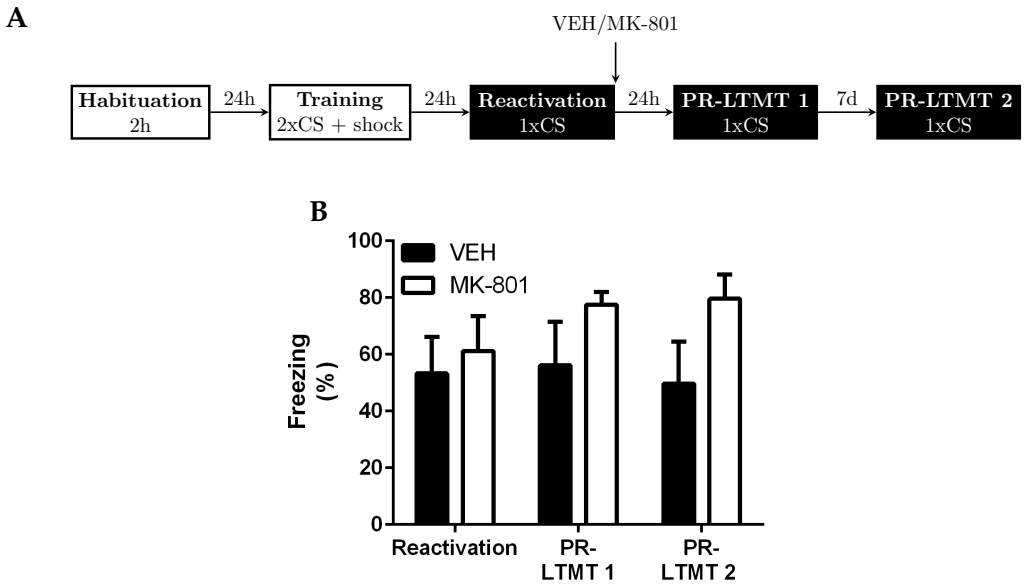


Figure 7.5: MK-801 administered after memory retrieval in a novel context had no effects of freezing in subsequent tests. **A**: Schematic of experimental procedures in Experiment 5. **B**: Freezing during the CS presentation in long-term memory tests. N=6 for both groups. Bars represent means +SEM.

Experiment 6: Effects of antagonism of NMDA receptors with MK-801 before a retrieval session consisting of 2 CS presentations

Whilst the manipulations of contextual information at reactivation were designed to increase the likelihood that there was sufficient novelty to warrant memory destabilisation it was possible that this did not deviate sufficiently from training to trigger reconsolidation. With this in mind, in Experiment 7 the reactivation session consisted of two CS presentations, each without shock. Whether reconsolidation was taking place was probed with pre-reactivation MK-801 treatment (see Figure 7.6A).

MK-801 treatment decreased freezing to each of the CS presentations in the reactivation session. However, this treatment had no effect on memory expression in either of PR-LTMTs (Figure 7.6B). This pattern of results was substantiated by a significant Session*Drug interaction ($F_{3,31.8} = 16.32$,

$p < .001$), a main effect of Session ($F_{3,31.8} = 8.81$, $p = .001$) but no main effect of Drug ($F_{1,16} = 2.34$, $p = .146$). Analysis of the significant Session*Drug interaction revealed a decrease in freezing in MK-801 treated rats at reactivation but at no other time points (Figure 7.6B).

None of the effects, or lack thereof, could be attributed to differences in freezing in the training sessions (Table 7.4) or before the CS was presented (Table 7.5).

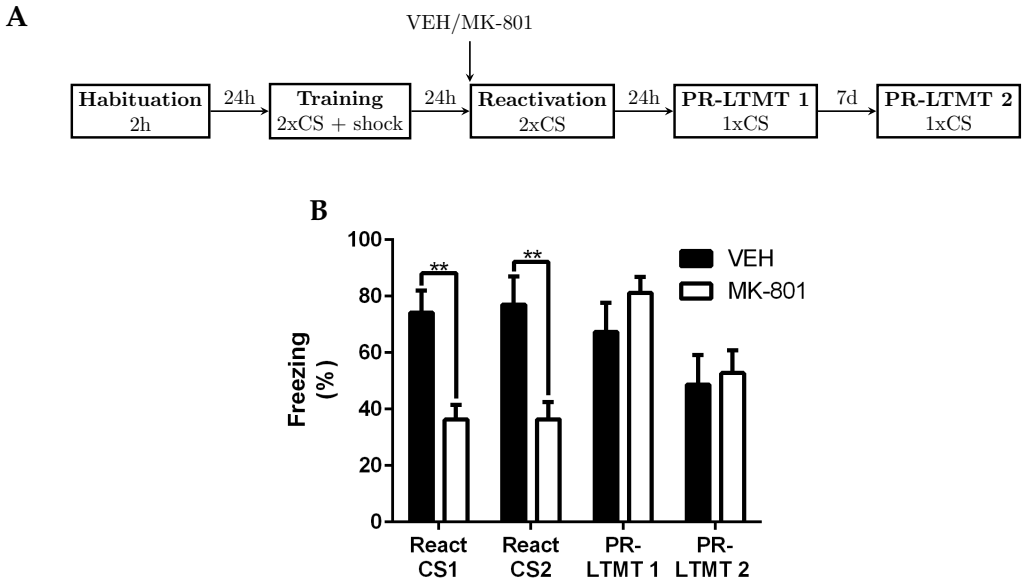


Figure 7.6: MK-801 administered before memory retrieval consisting of 2 CS presentations had no effects of freezing in subsequent tests. **A**: Schematic of experimental procedures in Experiment 6. **B**: Freezing during the CS presentation in long-term memory tests. N=9 for both groups. Bars represent means +SEM.

Experiment 7: Effects of antagonism of NMDA receptors with MK-801 before retrieval of a weakly trained fear memory

One potential boundary condition for reconsolidation to occur is memory strength (Wang *et al.*, 2009). In order to investigate whether this was preventing reconsolidation from taking place a group of animals were trained with a single CS-US pairing (see Figure 7.7A) and whether this enabled the memory to reconsolidate following a single CS presentation investigated.

Treatment with MK-801 before the memory reactivation session resulted in an acute deficit in freezing but did not affect memory expression in a PR-LTMT the next day (Figure 7.7B). This effect was substantiated by a significant Session*Drug interaction ($F_{1,10} = 5.29$, $p = .044$), with no main effect of Drug or Session (both: $F_{1,10} < 1.82$, $p > .206$).

It was not possible to directly compare levels of training between groups since only 1 CS was presented in this session, although baseline freezing to the CS was similar between groups (Table 7.4). Freezing prior to CS presentation was unaltered by MK-801 treatment before memory reactivation or test session (Table 7.5).

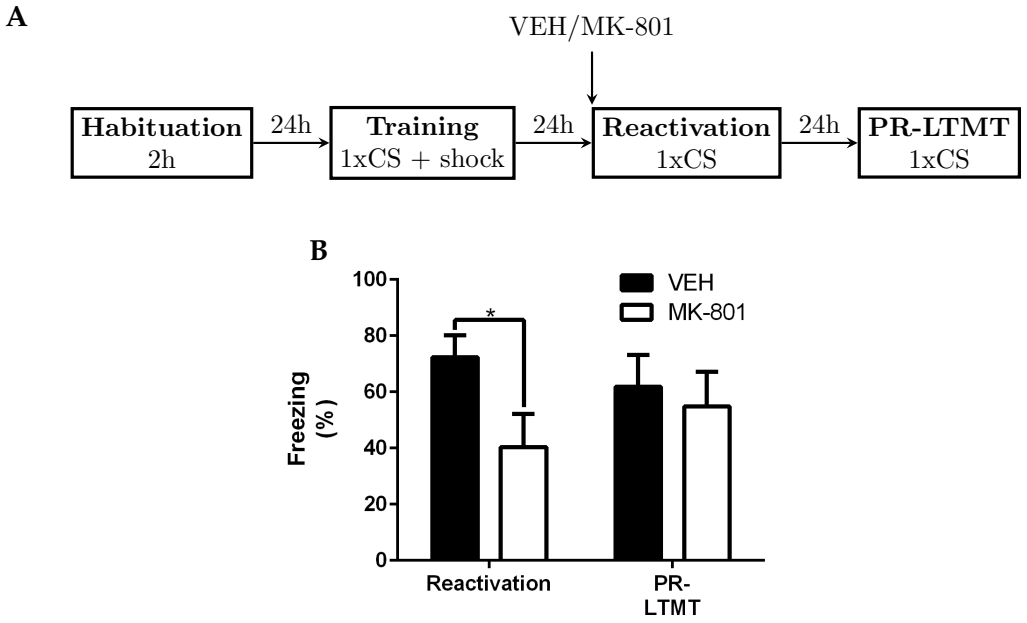


Figure 7.7: MK-801 administered before memory retrieval in animals given a single CS-US at training did not affect freezing the next day. **A**: Schematic of experimental procedures in Experiment 7. **B**: Freezing during the CS presentation in memory retrieval sessions. N=6 for both groups. Bars represent means +SEM.

Experiment 8: Effects of antagonism of NMDA receptors with MK-801 during acquisition

Despite parametric manipulation of the reactivation and training conditions, none of the previous experiments had been able to disrupt memory expression with NMDA receptor antagonism prior to, or following memory reactivation. With this in mind, Experiment 8 assessed the ability of MK-801 to prevent memory consolidation, rather than reconsolidation. MK-801 was administered prior to fear memory acquisition (see Figure 7.8A).

There was no evidence that MK-801 was able to prevent consolidation of an auditory fear memory. Treatment with MK-801 resulted in an acute deficit in freezing behaviour during the training session, with animals treated with MK-801 demonstrating similar levels of freezing during CS1 but this was decreased during CS2 (Figure 7.8B). These effects were indicated by an overall effect of Drug ($F_{1,9} = 22.05$, $p = .001$) and a significant Drug*CS interaction: ($F_{1,9} = 31.62$, $p < .001$). There was no

evidence of decreased freezing in MK-801 treated animals in either of the subsequent LTMTs (Figure 7.8C). This was indicated by no overall effect of Drug ($F_{1,9} < 0.01$, $p = .951$) and no significant Drug*Test interaction ($F_{1,9} = 0.28$, $p = .607$). There was also no effect of drug treatment on freezing before the CS was presented during these test sessions (Table 7.5).

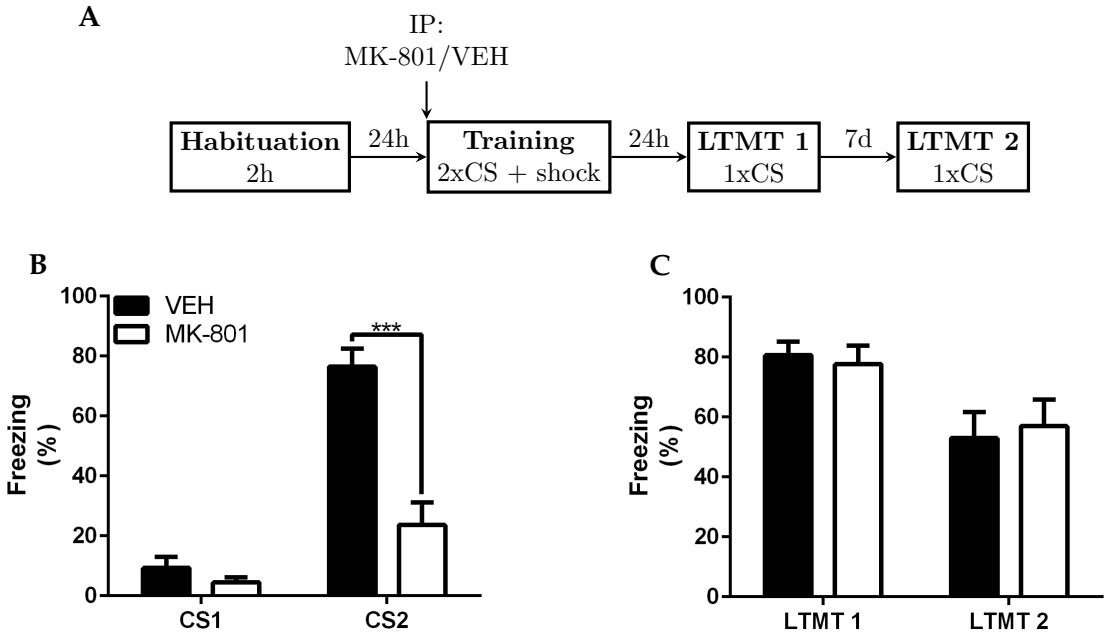


Figure 7.8: MK-801 administered before training of a CS-US association resulted in acute, but not long lasting effects on fear expression **A**: Schematic of experimental procedures in Experiment 8. **B**: Freezing during the CS presentation during training. **C**: Freezing during the CS presentation in memory retrieval sessions. N=5/6 per treatment group. Data are represented as means +SEM. *** p<.001

Experiment 9: Effects of antagonism of NMDA receptors with MK-801 prior to fear memory acquisition in naïve or pre-trained animals

Experiment 8 demonstrated that administration of the NMDA receptor antagonist MK-801 had no effect on the acquisition of a discrete fear association in animals that had previously undergone appetitive training. Whether this pre-training was responsible for the insensitivity of consolidation to NMDA receptor antagonism was investigated in a group of experimentally naïve animals. The effect of pre-training to modulate the requirement for NMDA receptor activation for memory acquisition was further investigated with a group who were trained to fear a light CS before auditory fear CS training took place (see Figure 7.9A).

Freezing to the auditory CS

In the auditory fear memory training sessions treatment with MK-801 resulted in a decrease in freezing regardless of pre-training, but only naïve animals treated with this drug increased their freezing between CS1 and CS2. These effects were supported by an overall effect of CS ($F_{1,20} = 74.72$, $p < .001$) and this effect differing between pre-training ($F_{1,20} = 5.16$, $p = .034$) and drug ($F_{1,20} = 34.01$, $p < .001$) groups. Crucially, there was also a CS*Pre-Training*Drug interaction ($F_{1,20} = 4.73$, $p = .042$). Subsequent analyses of this effect revealed that whilst treatment with MK-801 resulted in a decrease in freezing during training in both pre-trained and naïve animals ($t_{10} > 2.24$, $p < .049$), the only MK-801 treated animals that increased their freezing from CS1 and CS2 were those that were naïve (naïve: $t_5 = 2.81$, $p = .038$; pre-trained: $t_5 = 0.86$, $p = .427$; Figure 7.9B).

In the LTMT sessions pre-training increased fear expression occurring as a result of presentation of the auditory CS. However, the drug treatment administered prior to the pairing of this CS with shock had no effect on memory expression either 1 day or 1 week following training, suggesting MK-801 was unable to prevent fear memory consolidation. This was true regardless of whether animals had undergone pre-training or not. Overall levels of freezing were not affected by drug treatment ($F_{1,20} = 1.13$, $p = .302$) and this did not vary between pre-trained and naïve animals (Pre-training*Drug: $F_{1,20} = 0.03$, $p = .871$). Animals that were trained to fear a light CS showed increased freezing to the auditory CS in the test sessions ($F_{1,20} = 9.69$, $p = .005$). Freezing varied between the two test sessions ($F_{2,20} = 4.37$, $p = .050$) but this did not interact with any other factor (all: $F_{1,20} < 0.77$, $p > .389$, Figure 7.9C).

There was no evidence of altered associations between the context and shock, as demonstrated by similar levels of freezing before the CS was presented in all both LTMTs consisting of auditory CS presentation. Drug treatment before (auditory CS) training did not affect subsequent pre-CS freezing ($F_{1,20} = 0.40$, $p = .550$) and this did not vary between different pre-treatment groups (Pre-training*Drug: $F_{1,20} = 0.57$, $p = .458$). Finally, pre-CS freezing did not very between test sessions ($F_{1,20} = 0.53$, $p = .475$) and this was equally true regardless of drug treatment and pre-training groups (all interactions: $F_{1,20} < 3.87$, $p > .063$; Table 7.2)

CS modality	Auditory		Visual
Group	LTMT 1A	LTMT 2A	LTMT B
Pre-trained:			
VEH	0.4 ± 0.42	0.6 ± 0.24	1.4 ± 0.69
MK-801	0.7 ± 0.57	2.4 ± 1.35	13.8 ± 8.76
Naïve:			
VEH	0.2 ± 0.15	15.3 ± 13.18	0.7 ± 0.34
MK-801	0.5 ± 0.43	13.2 ± 12.13	1.4 ± 0.46

Table 7.2: Freezing before CS presentation in Experiment 9. Values represent mean values ± SEM to two decimal places.

Freezing to the visual CS

Animals in the pre-trained group successfully acquired the association between the light and shock delivery, as demonstrated by an increase in freezing to this stimulus in the training and a subsequent LTMT session. Animals (in the pre-trained group) increased their freezing between the two visual stimulus presentations within the training sessions ($F_{1,10} = 34.45$, $p < .001$), providing a within-subjects measure of learning. Prospective drug treatment groups did not affect acquisition of this association (CS*Drug: $F_{1,10} = 0.50$, $p = .826$) nor did they affect the overall level of freezing in the session (Drug: $F_{1,10} < 0.01$, $p = .996$; Figure 7.9D).

The increase in fear from CS1 to CS2 in pre-training was not purely the result of post-shock freezing. In a LTMT session consisting of light CS presentation animals that underwent light-shock conditioning showed increased fear to the visual CS in comparison to those that did not (Pre-training: $F_{3,20} = 11.56$, $p = .003$). Freezing during this test was not affected by drug treatment before auditory fear conditioning ($F_{1,20} = 1.06$, $p = .317$) and this effect was not modulated by pre-training (Pre-training*Drug: $F_{1,20} = 1.15$, $p = .297$; Figure 7.9E). Differences in freezing in pre-trained groups could not be explained by increased associations between the context and the shock in these animals; pre-training did not affect freezing before the light CS was presented ($F_{1,20} = 2.19$, $p = .154$) and this was true regardless of drug treatment (Pre-training*Drug: $F_{1,20} = 1.75$, $p = .201$). Overall levels pre-CS freezing were not affected by the drug administered before the auditory fear training sessions ($F_{1,20} = 2.21$, $p = .153$; Table 7.2).

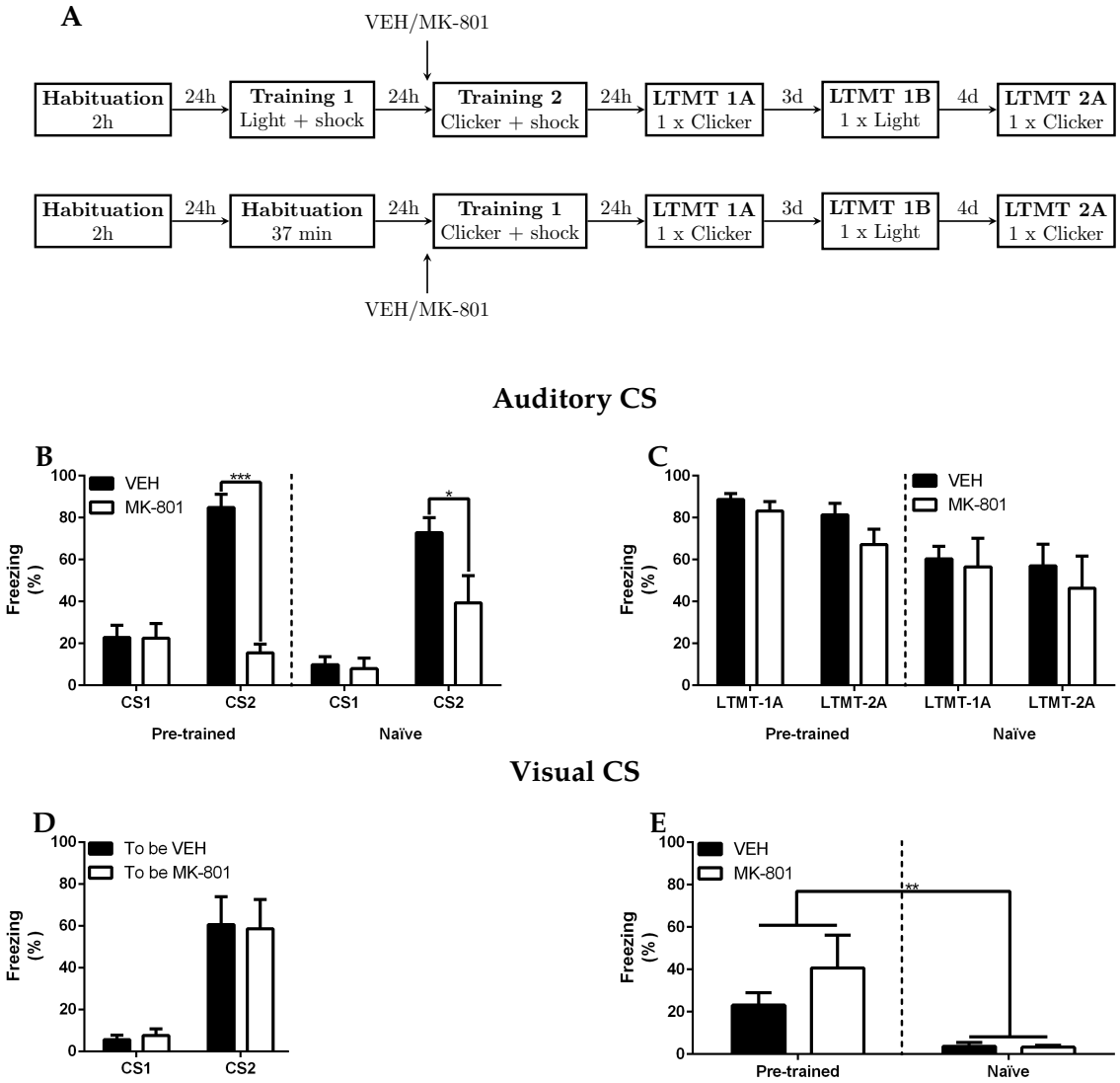


Figure 7.9: MK-801 administered before training of a CS-US association resulted in acute, but not long lasting effects on fear expression, regardless of pre-training. **A**: Schematic of experimental procedures in Experiment 9. **B**: Freezing during the auditory CS presentation during training. **C**: Freezing during LTMTs to the presentation of the auditory CS. **D**: Freezing during the light CS during pre-training. **E**: Freezing during the light CS presentation in LTMT 1B. N=6 in each treatment group. Data are represented as means +SEM.

Experiment 10: Effects of inhibition of the mTOR pathway following retrieval

Owing to the failure to prevent both memory reconsolidation and consolidation with NMDA receptor antagonism subsequent experiments targeted processes downstream of the activation of these receptors. Rapamycin prevents mTOR signalling, which is required for synaptic plasticity, is activated downstream of NMDA receptor activation (Hoeffer and Klann, 2010) and is known to be required for reconsolidation to take place (Blundell *et al.*, 2008; Gafford *et al.*, 2011; Hoffman *et al.*, 2015). Animals were injected with rapamycin immediately following the memory reactivation session. In order to investigate the potential state-dependency of the resultant decrease in fear expression animals also underwent a third memory test, with rapamycin injections being administered to all animals prior to this session (Gisquet-Verrier and Riccio, 2012; Gisquet-Verrier *et al.*, 2015; Figure 7.10A).

Animals treated with rapamycin following memory retrieval demonstrated equal levels of freezing during both the reactivation and first PR-LTMT session. However, 8 days after drug administration animals demonstrated a profound reduction in freezing toward the CS (Figure 7.10B). This suggested a delay-dependent effect of rapamycin to produce a deficit in fear memory expression. Treatment with rapamycin prior to a third test session 3d later resulted in a loss of the effect of rapamycin treatment, suggesting that the deficit detected in PR-LTMT2 was the result of state-dependent learning. The differential effect of rapamycin during the different test sessions was qualified by a significant Drug*Session interaction ($F_{1.5,15.4} = 5.00$, $p = .028$) with no main effect of Drug ($F_{1,10} = 2.34$, $p = .157$) and an overall effect of Session ($F_{1.5,15.4} = 5.30$, $p = .024$). Simple effects analysis of this interaction only revealed a significant decrease in freezing in the memory test conducted 7 days after reactivation (Figure 7.10B).

None of the effects occurring in the test sessions could be attributed to pre-existing differences in freezing during training (Table 7.4) or freezing to the context in the periods before the CS was presented (Table 7.3).

Group	Reactivation	PR-LTMT 1	PR-LTMT 2	PR LTMT 3	Effect of drug	Drug*Test interaction
Vehicle	3.1 ± 0.57	6.6 ± 4.59	5.3 ± 1.9	16.5 ± 8.90	$F_{1,10} = 2.58$, $p = .140$	$F_{1.3,12.9} = 1.40$, $p = .269$
RAPA	0.1 ± 0.06	1.7 ± 1.01	1.9 ± 7.16	3.0 ± 2.27		

Table 7.3: Freezing during pre-CS periods of test sessions in Experiment 10. All values represent percentage of the minute before CS presentation spent freezing $\pm$ SEM to 2 decimal points.

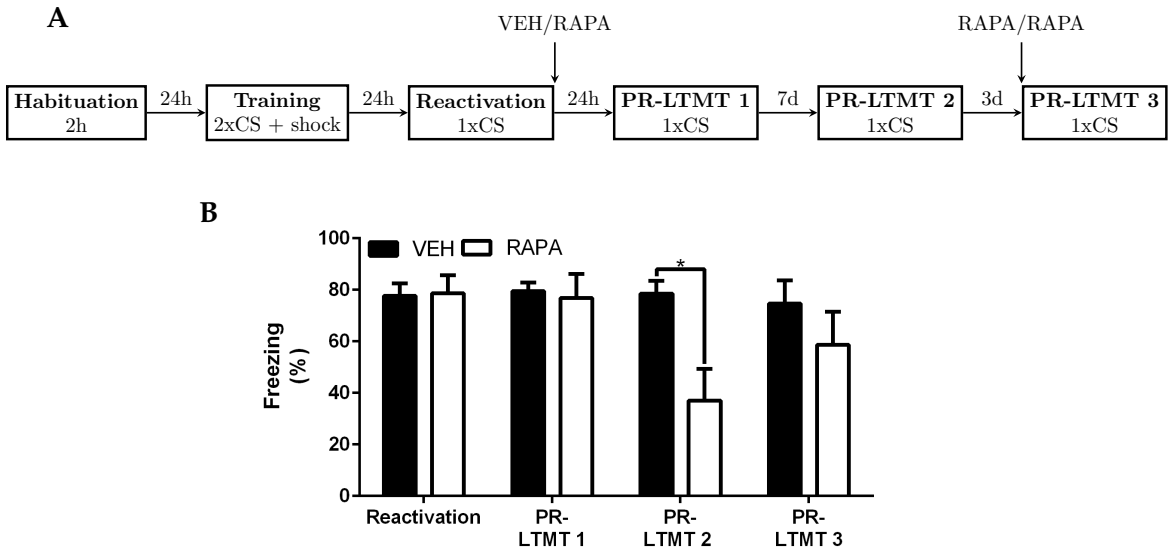


Figure 7.10: Rapamycin administered following memory retrieval resulted in delay-dependent amnesia that is reversed by pre-retrieval administration of this same drug. **A**: Schematic of experimental procedures in Experiment 9. **B**: Freezing during the CS presentation in memory retrieval sessions. N=6 for both groups. Bars represent means +SEM. * $p < .05$

Rapamycin treatment caused a considerable reduction in body weight (Figure 7.11), consistent with previous reports (Fifield *et al.*, 2013; Hebert *et al.*, 2014). There was an overall effect of Day on body weight ($F_{2.2,22.3} = 90.40$, $p < .001$), a significant Day*Treatment interaction ($F_{2.2,22.3} = 52.21$, $p < .001$) and a main effect of Drug ($F_{1,10} = 68.45$, $p < .001$). Separate analyses of the two treatment groups revealed that whilst body weight of both vehicle and rapamycin treated rats' weights varied between days ($F_{8,40} > 16.31$, $p < .001$), post-hoc tests revealed that animals treated with vehicle gained weight between reactivation and the following 4d ($p = .004$), whilst rapamycin treated rats lost weight in the same period ($p = .008$).

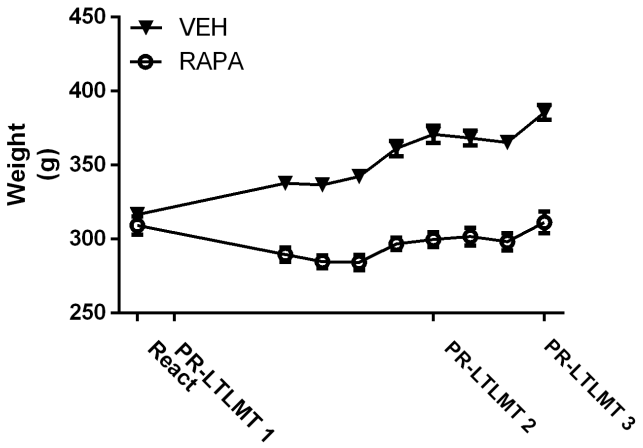


Figure 7.11: Rapamycin treatment resulted in long lasting weight loss. See text for details. N=6 for both groups. Values represent means ±SEM.

Experiment 11: Effects of intra-BLA anisomycin following memory retrieval

Given that previous experiments were unable to demonstrate any memory impairment with NMDA receptor antagonism and the deficits occurring as a result of rapamycin treatment appeared to be caused by state-dependent learning, this experiment attempted to prevent reconsolidation with intra-BLA infusion of the protein synthesis inhibitor anisomycin (see Figure 7.12A).

As with previous manipulations, post-reactivation anisomycin had no effect on freezing in later test sessions (Figure 7.12B). This was indicated by no main effect of Drug ($F_{1,18} = 0.49$, $p = .466$) and no Drug*Test interaction ($F_{2,36} = 0.70$, $p = .503$).

The interpretation of the data from the test sessions was not confounded by pre-existing differences in freezing levels of treatment groups during the training sessions (Table 7.4) or the periods before the CS was presented (Table 7.5).

Only animals with cannula tips that could be located within the BLA were included in the above analysis (Figure 7.13).

Power analysis

Whilst the difference in freezing between the two treatment groups during PR-LTMT1 was not significant, there was a trend for animals infused with anisomycin to show less fear as a result of CS presentation during this session. In order to investigate whether the failure to detect decreased freezing in these animals was due to a lack of statistical power, the effect size was calculated for

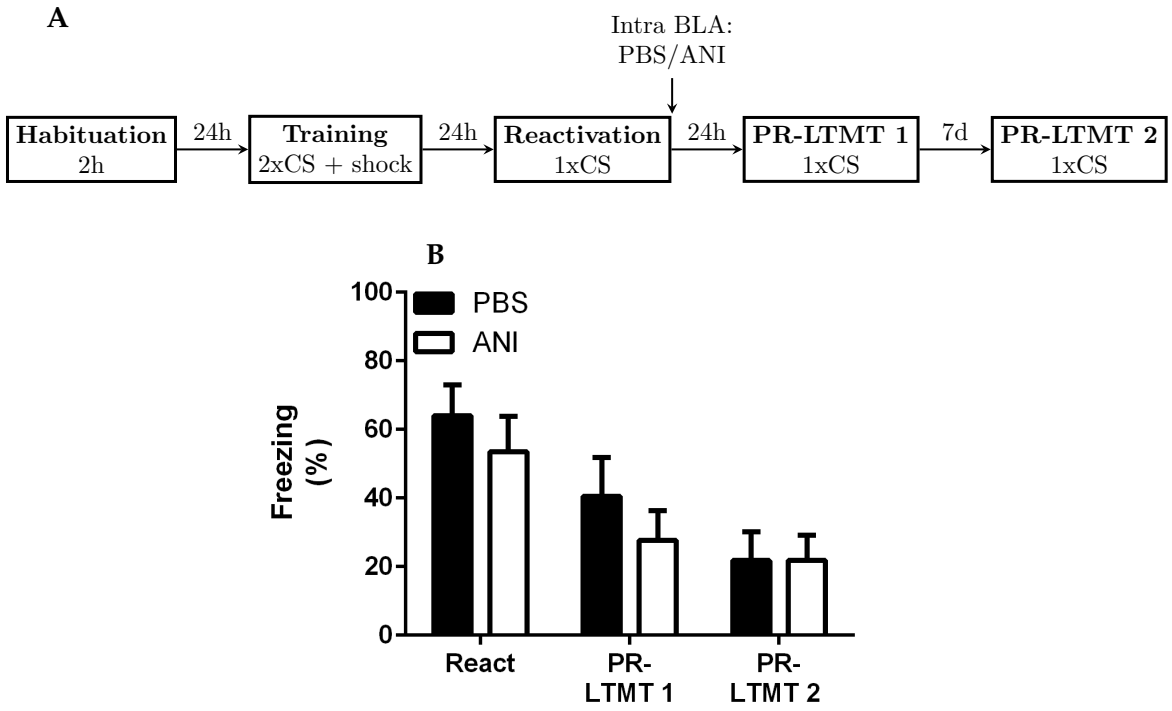
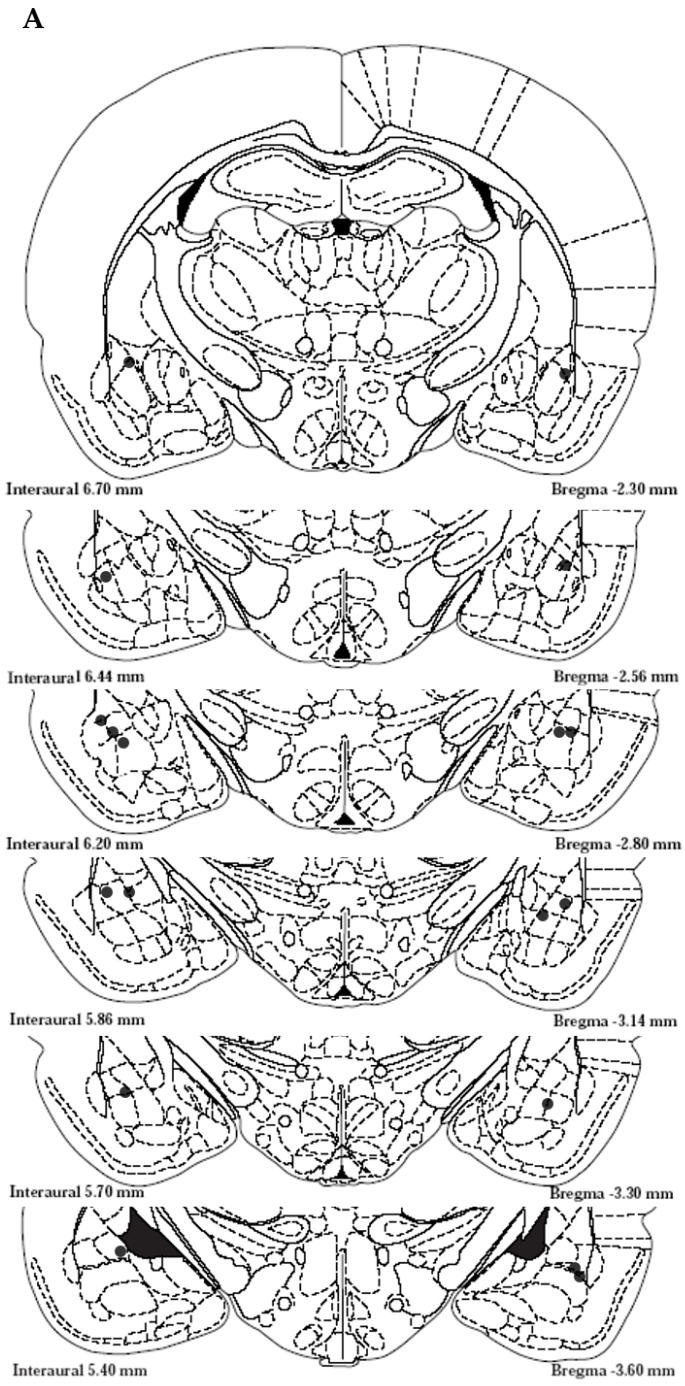


Figure 7.12: Intra-BLA infusion of the protein synthesis inhibitor anisomycin immediately following memory retrieval had no effect on subsequent fear memory expression. **A**: Schematic of experimental procedures in Experiment 10. **B**: Freezing during the CS presentation in memory retrieval sessions. Bars represent means +SEM. N=9/11 for each treatment group.

these data. The difference between the two treatment groups at PR-LTMT1[1] resulted in an effect size (d) of 0.41, representing a small to medium effect size (Cohen, 1992). In comparison, previous studies with similar methods have obtained effect sizes of approximately 0.79 (Nader *et al.*, 2000[2]). Further analysis revealed that Experiment 11 was indeed underpowered ($\eta^2 = 0.53$). A total of 190 rats (95 per group) would be required to achieve 80% power with p value of .05; this would not be practical and is a much higher n than has been used in previous studies of a similar nature (n$=7/8$ in Nader *et al.*, 2000, although this study only achieved power of approximately 29%; a total of 50 rats would be required to achieve this with an effect size of 0.41). Thus, whilst the possibility of a type II error cannot be excluded it does appear that the results obtained in the present experiment are at least quantitatively smaller than those previously reported (Nader *et al.*, 2000).

[1]In order to be certain that any differences in freezing are not the result of pre-existing differences between the two groups it would, in addition to a significant result in comparing these two groups, be necessary to yield a significant Test*Drug interaction. Conducting power analysis in order to calculate the number of subjects required for a t-test is a somewhat liberal approach and likely underestimates the number of animals that would be required in order to be certain any result obtained was due to the amnestic effects of post-reactivation anisomycin.

[2]Effect size estimated from Figure 2C, trial 1 of Nader *et al.* (2000).


A
Interaural 6.70 mm
Bregma -2.30 mm
Interaural 6.44 mm
Bregma -2.56 mm
Interaural 6.20 mm
Bregma -2.80 mm
Interaural 5.86 mm
Bregma -3.14 mm
Interaural 5.70 mm
Bregma -3.30 mm
Interaural 5.40 mm
Bregma -3.60 mm

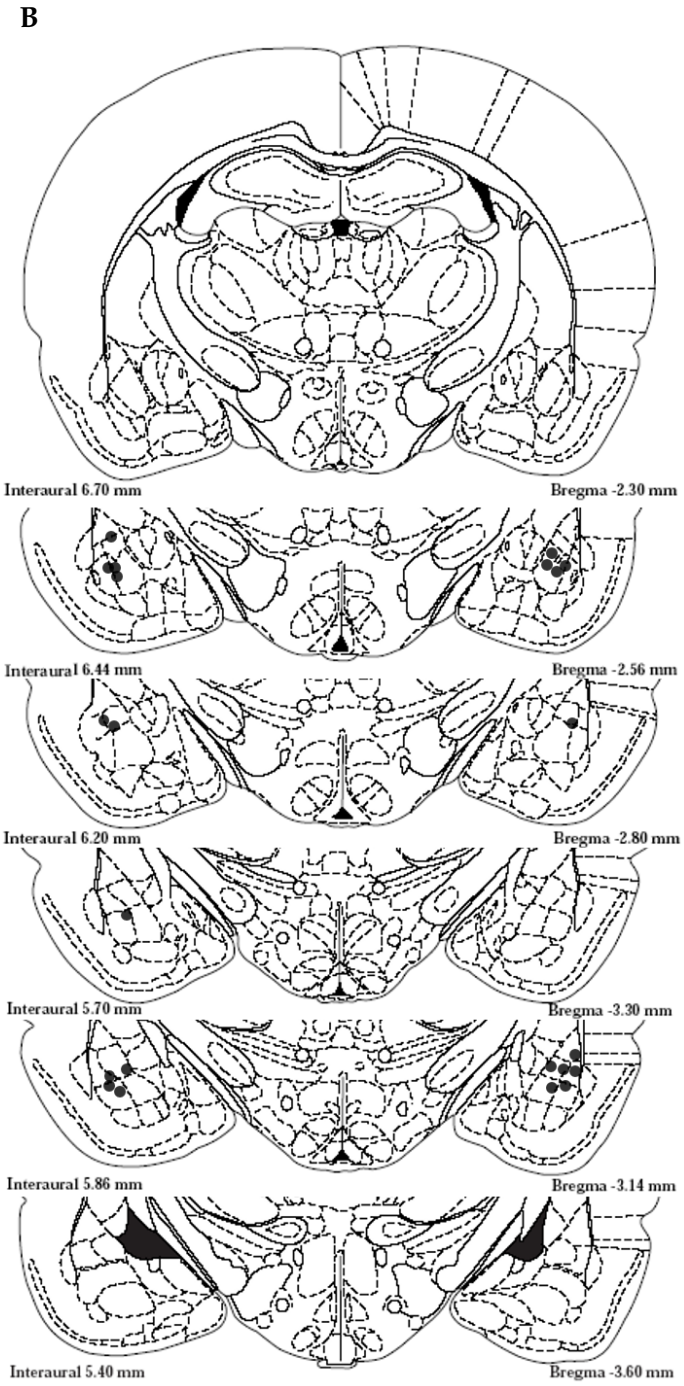


Figure 7.13: Approximate location of cannula tips for Experiment 11. **A**: Cannula tip locations for animals infused with PBS. **B**: Cannula tip locations for animals infused with anisomycin. Figure from Paxinos and Watson (1998).

Exp.	Training			
Group	CS1	CS2	Effect of Drug	Drug*CS interaction
1				
Vehicle	6.0 ± 1.58	67.1 ± 1.27	$F_{1,10} = 0.46$, $p = .511$	$F_{1,10} = 0.54$, $p = .479$
MK-801	6.0 ± 1.27	72.6 ± 3.61		
2				
Vehicle	9.8 ± 6.68	66.6 ± 7.39	$F_{1,10} = 0.87$, $p = .374$	$F_{1,10} = 0.15$, $p = .707$
CPP	13.17 ± 8.07	75.6 ± 5.67		
3				
Vehicle	8.4 ± 5.46	59.1 ± 9.36	$F_{1,10} = 0.02$, $p = .966$	$F_{1,10} = 0.12$, $p = .736$
MK-801	6.4 ± 2.61	61.9 ± 9.20		
4				
Vehicle	16.6 ± 3.48	59.2 ± 12.89	$F_{1,10} = 1.30$, $p = .280$	$F_{1,10} = 0.62$, $p = .451$
MK-801	3.1 ± 2.41	57.5 ± 4.24		
5				
Vehicle	8.9 ± 4.22	59.3 ± 13.69	$F_{1,10} = 2.65$, $p = .135$	$F_{1,10} = 0.00$, $p = .972$
MK-801	22.1 ± 8.63	73.2 ± 6.40		
6				
Vehicle	3.0 ± 1.84	66.9 ± 8.41	$F_{1,16} = 0.61$, $p = .447$	$F_{1,16} = 0.03$, $p = .863$
MK-801	5.0 ± 1.58	68.5 ± 5.87		
7				
Vehicle	5.2 ± 4.97		$F_{1,10} < 0.01$, $p > .999$	
MK-801	2.2 ± 1.46			
10				
Vehicle	22.2 ± 10.90	75.9 ± 5.90	$F_{1,10} = 1.11$, $p = .316$	$F_{1,10} = 0.12$, $p = .733$
Rapamycin	15.6 ± 10.56	64.4 ± 9.06		
11				
PBS	12.7 ± 5.63	70.6 ± 3.90	$F_{1,18} = 1.28$, $p = .273$	$F_{1,18} = 0.12$, $p = .979$
ANI	6.7 ± 1.94	64.5 ± 6.45		

Table 7.4: Freezing during training in experiments attempting to prevent reconsolidation. Exp.: Experiment. All values represent the average percentage of time of the scored period spent freezing $\pm$ SEM to 2 decimal places.

Exp. Group	Reactivation session	(PR-) LTMT 1	(PR-) LTMT 2	Effect of Drug	Drug*Test interaction
1					
Vehicle	1.1 ± 0.57	1.2 ± 0.60	0.2 ± 0.19	$F_{1,10} = 1.38$, $p = .268$	$F_{1.0,10.4} = 1.17$, $p = .306$
MK-801	2.0 ± 0.63	0.2 ± 0.15	8.1 ± 7.16		
2					
Vehicle	1.2 ± 0.54	2.2 ± 0.54	1.4 ± 0.89	$F_{1,10} = 0.42$, $p = .532$	$F_{1.1,11.5} = 1.89$, $p = .197$
CPP	5.1 ± 2.81	1.3 ± 1.09	1.1 ± 0.78		
3					
Vehicle	0.6 ± 0.46	1.1 ± 0.80		$F_{1,10} = 0.41$, $p = .538$	$F_{1,10} = 0.44$, $p = .522$
MK-801	0.4 ± 0.33	3.1 ± 2.83			
4					
Vehicle	0.6 ± 0.25	5.4 ± 1.82	4.8 ± 1.70	$F_{1,10} = 0.98$, $p = .346$	$F_{2,20} = 5.75$, $p = .011$
MK-801	5.5 ± 2.88	0.7 ± 0.45*	0.9 ± 0.42*		
5					
Vehicle	1.8 ± 0.70	$1.3. \pm 0.42$	2.4 ± 1.74	$F_{1,10} = 2.27$, $p = .163$	$F_{2,20} = 0.16$, $p = .851$
MK-801	7.1 ± 2.49	6.4 ± 4.74	6.0 ± 2.84		
6					
Vehicle	0.2 ± 0.70	5.3 ± 4.96	3.6 ± 2.69	$F_{1,10} = 2.27$, $p = .163$	$F_{2,20} = 0.16$, $p = .851$
MK-801	1.5 ± 0.43	0.1 ± 0.09	0.5 ± 0.18		
7					
Vehicle	0.3 ± 0.15	0.9 ± 0.64		$F_{1,10} < 0.01$, $p > .962$	$F_{1,10} = 4.22$, $p = .067$
MK-801	0.9 ± 0.35	0.3 ± 0.20			
8					
Vehicle		1.7 ± 1.12	0.6 ± 0.34	$F_{1,10} = 2.27$, $p = .163$	$F_{2,20} = 0.16$, $p = .851$
MK-801		0.3 ± 0.16	0.4 ± 0.25		
11					
PBS	0.6 ± 0.26	6.5 ± 3.66	0.7 ± 0.43	$F_{1,18} = 0.91$, $p = .354$	$F_{1.2,21.4} = 1.15$, $p = .246$
ANI	1.3 ± 0.99	2.3 ± 1.29	0.5 ± 0.26		

Table 7.5: Freezing during pre-CS periods of retrieval sessions in experiments using NMDA receptor antagonism to attempt to prevent (re)consolidation. All values represent percentage of the minute before CS presentation spent freezing $\pm$ SEM to 2 decimal places. *$p < .05$ compared to vehicle.

Discussion

Summary of results

It was not possible to disrupt reconsolidation with NMDA receptor antagonism or protein synthesis inhibition. This was despite the use of two different NMDA receptor antagonists, altering the timing of drug administration, providing novel contextual information during the reactivation session and altering the number of CSs presented during the reactivation and training sessions. A subsequent experiment demonstrated that MK-801 was unable to prevent memory consolidation, suggesting that the failures to block reconsolidation may have been attributed to a failure in the amnestic agent to do so, rather than this process not taking place. Protein synthesis inhibition was also ineffective at preventing memory reconsolidation suggesting that the retrieval conditions used here were also insufficient to result in memory destabilisation required for reconsolidation to take place.

Relationship to previous work

NMDA receptor antagonism with MK-801 prior to retrieval of a consolidated association between an auditory CS and footshock has previously been reported to result in decreased fear to the reactivated CS in subsequent tests (Lee *et al.*, 2006b; Merlo *et al.*, 2014). Using near almost identical training, reactivation and testing conditions it was not possible to replicate this effect. In fact, when tested a week after memory reactivation, treatment with MK-801 resulted in *increased* subsequent fear expression. The reason for this contradiction of previous literature is unclear but is not the first report of memory enhancing effects of NMDA receptor antagonism. Pre, but not post-reactivation treatment with ketamine has previously been reported to result in memory enhancements (Honsberger *et al.*, 2015). This effect was speculatively attributed to the lower affinity of ketamine on NMDA receptors resulting in disinhibition of cortical networks (Murray *et al.*, 2014); treatment with memantine, an NMDA receptor antagonist with even lower activity on these receptors, also appears to enhance the reconsolidation of avoidance memories in the day old chick (Samartgis *et al.*, 2012). This would suggest that the memory enhancing effects of MK-801 may have been the result of a reduced affinity of this drug on NMDA receptors in comparison to previous studies.

One possible explanation for the increased freezing in animals treated with MK-801 in Experiment 1 is that this drug was preventing extinction taking place in the retrieval session. It is known this process is NMDA receptor-dependent, as indicated by increased freezing in drug-treated groups

the day after extensive presentation of the CS without shock (Lee *et al.*, 2006b; Merlo *et al.*, 2014; Santini *et al.*, 2001). However, this explanation is unlikely, primarily because there was no evidence of a decrease in freezing between the memory reactivation and test session, as would be expected if this were the case. Furthermore, it is unlikely that a single CS would result in extinction; 4 non-reinforced CS presentations does not trigger this process in memories trained in a similar fashion (Merlo *et al.*, 2014).

Whilst there is evidence that NMDA receptor antagonism can prevent reconsolidation (Lee *et al.*, 2006b; Merlo *et al.*, 2014; Milton *et al.*, 2008a), studies have also reported that administration of these drugs can prevent destabilisation, as indicated by their ability to protect against the amnestic effects of post-reactivation infusions of the protein synthesis inhibitor anisomycin (Ben Mamou *et al.*, 2006; Yu *et al.*, 2016). Although this would not typically be expected to result in memory enhancements (Ben Mamou *et al.*, 2006; Lee and Flavell, 2014; Lee *et al.*, 2008; Milton *et al.*, 2013), this might explain the lack of amnestic effects of MK-801 treatment both in this and previous chapters.

In Experiment 2 MK-801 was administered immediately following reactivation. This should enable the memory to become destabilised during the session, but prevent the restabilisation of the memory trace following it. Whilst the majority of studies preventing reconsolidation with NMDA receptor antagonists have administered these drugs prior to memory reactivation (e.g. Exton-McGuinness *et al.*, 2014; Lee *et al.*, 2006b; Milton *et al.*, 2008a), there is some evidence suggesting that post-reactivation treatment can also prevent restabilisation (Lee and Flavell, 2014; Przybyslawski and Sara, 1997; Tedesco *et al.*, 2014b). Administration of MK-801 following memory reactivation was not able to prevent reconsolidation; animals showed equal fear to the CS regardless of drug treatment.

One issue with post-reactivation administration of NMDA receptor antagonists is that the specific stage of reconsolidation in which activation of these receptors is required is unclear. NMDA receptor activation occurs upstream of the expression of *zif-268* for reward-related memories (Milton *et al.*, 2008a) and of ERK expression in fear memory reconsolidation (Merlo *et al.*, in preparation; also see Cammarota *et al.*, 2000). One possibility is that even in the short time taken to remove the animal from the chamber, the window in which NMDA receptor activation is required for reconsolidation to occur had already closed. Further investigation of the destabilisation-preventing ability of NMDA receptor antagonists has revealed that these effects are likely mediated by the action of these drugs on the GluN2B subunit of the NMDA receptor, whilst antagonism of the GluN2A subunit prevents reconsolidation (and not destabilisation) (Milton *et al.*, 2013). With this in mind, a

different NMDA receptor antagonist, CPP, was used. This drug has been shown to have slightly higher affinity to GluN2A receptors (Feng *et al.*, 2004; Feng *et al.*, 2005), and thus may be less likely to prevent destabilisation even if given pre-reactivation. Indeed, previous studies have demonstrated impairments in memory reconsolidation with this compound when it is given at this time point (Suzuki *et al.*, 2004). This treatment was not effective at preventing memory reconsolidation, although unlike pre-reactivation MK-801 treatment there was no evidence of a memory enhancement with this drug.

One of the major hurdles in interpreting an inability to produce amnesia whilst attempting to prevent reconsolidation is that it is unclear whether the result is due to a failure of the drug to prevent reconsolidation, or whether the retrieval conditions are insufficient to reactivate the memory. Given the previous literature suggesting that MK-801 prevents reconsolidation (Lee *et al.*, 2006b; Merlo *et al.*, 2014), it was possible that the pattern of results could be explained by the memory retrieval session not resulting in reconsolidation, rather than an inability to prevent this process with NMDA receptor antagonism. With this in mind, in Experiment 4 the retrieval session was manipulated in an attempt to increase the likelihood of destabilisation occurring. One of the key determinants of whether a reactivation session will result in the destabilisation of a memory is prediction error (PE) (Pedreira *et al.*, 2004; Sevenster *et al.*, 2013) and likely relates to the function of reconsolidation to integrate new information into existing memories (Lee *et al.*, 2009; Nader and Einarsson, 2010). The experiments described thus far attempted to generate this with the surprising absence of an anticipated reinforcer. However, it is possible that this alone was insufficient to result in memory destabilisation.

Recent experiments in fear memories have demonstrated that only when memory reactivation occurs in a novel context is there an increase in proteasome and GluR2 levels in the BLA (Jarome *et al.*, 2015), two markers that have been implicated in memory destabilisation (Jarome *et al.*, 2012). Furthermore, only when reactivation was conducted in a novel context was intra-BLA anisomycin effective at reducing fear expression in later tests (Jarome *et al.*, 2015). It is possible that the experience of the CS within a novel context, combined with the absence of a shock, but not the absence of the shock alone, was sufficient for reconsolidation to take place. With this in mind, in Experiment 4 MK-801 was administered before a reactivation session that was conducted in a similar fashion as before, but occurred in a context that the animals had not previously experienced. This was apparently ineffective at triggering reconsolidation; MK-801 treated animals demonstrated equal levels of freezing in all tests conducted after the reactivation session.

As mentioned previously, one issue in administering MK-801 prior to memory reactivation is that it may prevent destabilisation (Yu *et al.*, 2016). It was possible that although the shift in context meant that retrieval conditions were sufficient to trigger reconsolidation, MK-801's effect on GluN2B receptors (Wong *et al.*, 1986; Wong *et al.*, 1988) prevented memory destabilisation taking place (Ben Mamou *et al.*, 2006; Milton *et al.*, 2013). With this in mind, in Experiment 5 MK-801 was given immediately following memory reactivation in a novel context. Once again, however, NMDA receptor antagonism failed to result in any amnestic effect under these conditions.

As previously discussed, a major factor in determining whether a retrieval session results in destabilisation of the memory trace is the presence of novel information (Lee, 2009; Pedreira *et al.*, 2004; Sevenster *et al.*, 2013). Whilst prior experiments attempted to maximise this with a shift in context, Experiment 6 increased the degree of PE by presenting a larger number of CSs (in the absence of shock delivery) within the memory reactivation session. Reactivations consisting of prolonged CS exposure can lead to memory destabilisation where shorter sessions are unable to do so (Inda *et al.*, 2011; Reichelt and Lee, 2013a; Suzuki *et al.*, 2004). Increasing the number of CSs during the reactivation session was also ineffective at triggering reconsolidation that was susceptible to treatment with MK-801.

The strength of a memory can affect its ability to undergo reconsolidation (Inda *et al.*, 2011; Kwak *et al.*, 2012; Reichelt and Lee, 2013a; Suzuki *et al.*, 2004; Wang *et al.*, 2009). Although the experiments described used similar protocols and shock intensities as has been used in previous studies (Lee *et al.*, 2006b; Merlo *et al.*, 2014), it was possible that subtle procedural differences resulted in the formation of a stronger memory. Visual inspection of the mean level of freezing between Lee *et al.* (2006b) and the present experiments suggests this may be the case (although similar levels of freezing as in the present experiments were reported in Merlo *et al.*, 2014). Furthermore, previous experiments demonstrating fear memory reconsolidation impairments have used a single CS-shock pairing (albeit at a higher intensity), rather than the two used in the present experiments (e.g. Duvarci *et al.*, 2005; Milton *et al.*, 2013; Nader *et al.*, 2000). Several studies have shown that stronger memories are more resistant to reconsolidation, and although in some cases stronger memories can be destabilised with longer reactivation sessions (Suzuki *et al.*, 2004), this is not always effective (Wang *et al.*, 2009). With this in mind, Experiment 7 used a weaker training protocol; the CS was paired with shock once and memory reactivation sessions conducted as before. Increased memory strength did not appear to be the factor that was preventing reconsolidation taking place since pretreatment with MK-801 prior to the memory reactivation session was without effect on long-term memory (LTM) expression.

The majority of the manipulations described up to this point focussed on optimising the behavioural parameters of the reactivation session in order to maximise the likelihood of reconsolidation taking place. In evaluating the efficacy of these manipulations it was assumed that the amnestic agent used, MK-801 (in all but Experiment 3), would be effective at preventing reconsolidation, should it be taking place. Given the numerous parameters of the reactivation and training sessions that had been manipulated, each apparently without effect, the possibility remained that the tool used to probe whether reconsolidation taking place was ineffective, rather than the reactivation conditions themselves.

In order to independently confirm the efficacy of MK-801 to prevent reconsolidation a reactivation session was required that would, without any uncertainty, result in destabilisation. Since this was not available, the ability of MK-801 to prevent consolidation was investigated; the increase in fear between the first time the CS is presented during training and the test session 1 day later provides direct evidence that memory consolidation is taking place. MK-801 was also ineffective at preventing this memory process. Whilst there was an acute reduction in freezing during the training sessions in response to MK-801 treatment, likely the result of the effect of this drug to increase locomotor activity (Frantz and Hartesveldt, 1999), the following day there was no evidence of an amnestic effect.

It has been previously reported that learning can take place in the absence of NMDA receptor activation. This has been reported in contextual fear (Hardt *et al.*, 2009; Sanders and Fanselow, 2003; Wiltgen *et al.*, 2010), the Morris water maze (Bannerman *et al.*, 1995; Saucier and Cain, 1995) and extinction of discrete auditory fear associations (Langton and Richardson, 2008; Langton and Richardson, 2010). However, in each case this only occurs when there has been prior learning of a similar association before attempts are made to block subsequent learning with NMDA receptor antagonism. Little is known with regard to the similarity of two learning episodes required to render the second NMDA receptor independent. In Experiment 8 animals had undergone prior appetitive training which may have resulted in subsequent learning occurring in the absence of NMDA receptor activation. Experiment 9 addressed this issue, using experimentally naïve animals. The possibility of prior learning affecting the susceptibility of subsequent fear memory acquisition to NMDA receptor antagonism was also investigated with the inclusion of a group of animals that were trained to fear a light stimulus before auditory fear conditioning took place. However, it appeared that the prior training was not the sole contributor of the insensitivity of learning to NMDA receptor antagonism; fear memory acquisition was unimpaired by MK-801 treatment in animals in Experiment 9, regardless of whether they had undergone pre-training.

It appeared that NMDA receptor activation was not required for acquisition of fear memories, raising the possibility that in several of the experiments conducted previously reconsolidation may have taking place, but without the need for NMDA receptor activation. Several reasons for these effects, and why there has been an apparent loss of reconsolidation effects once reported are now discussed.

One possibility was that MK-801 was not effective at antagonising NMDA receptors and it is for this reason it was unable to prevent reconsolidation. However, this is improbable, since this drug has been used extensively in reconsolidation studies (e.g. Exton-McGuinness *et al.*, 2014; Lee *et al.*, 2006b; Merlo *et al.*, 2014; Milton *et al.*, 2008a; Wouda *et al.*, 2010). It is unlikely that the results were due to quality control issues in the production line of this drug, given that recent studies in the lab have used two different suppliers of MK-801 with similar effects (Abcam: experiments in this chapter, Sigma: experiments in prior chapters, Merlo *unpublished observations*, Cahill *unpublished observations*). Studies using higher doses of this drug (0.3mg/kg) are also ineffective at preventing reconsolidation using conditions once able to result in reconsolidation (Cahill, *unpublished observations*, *cf.* Merlo *et al.*, 2014). It should also be noted that in the majority of experiments conducted MK-801 treatment resulted in an acute decrease in fear expression, consistent with its ability to increase locomotor activity (Frantz and Hartesveldt, 1999) confirming the injections were successful and the drug was in solution. It is therefore, highly unlikely the results of this chapter were caused by a lack of pharmacological efficacy of this drug.

A third possibility is that some subtle aspect of the reactivation sessions was different from previous studies. This is not likely since the equipment used in the present experiments is the same as used in previous reports (Lee *et al.*, 2006b; Merlo *et al.*, 2014). The results are unlikely the result of differences in the way these experiments are conducted since reconsolidation deficits have eluded researchers once able to produce such effects (Merlo, *unpublished reports*, *cf.* Merlo *et al.*, 2014). This explanation is also unable to explain why NMDA receptor antagonism could not prevent fear memory consolidation.

This only leaves the possibility that some aspect of the animals themselves is different from before. For example, some aversive experience may occur before animals were delivered, rendering subsequent learning NMDA receptor-independent (Hardt *et al.*, 2009; Sanders and Fanselow, 2003; Wiltgen *et al.*, 2010). Alternatively the use of different ages of rats may alter NMDA receptor expression (Armentia and Sah, 2003; Monyer *et al.*, 1994; Sheng *et al.*, 1994), resulting in differences in the sensitivity of manipulations of these drugs. Although subjects used in the present experiments

were of similar weights to previous studies, potential differences in diet may have meant whilst they were of a similar mass, the ages may have been different.

Whilst it has not been explicitly tested, it is likely that NMDA receptor independent learning requires the synthesis of new proteins: long-term potentiation (LTP) occurring in the absence of activation of these receptors is blocked by anisomycin (Moosmang *et al.*, 2005). It was possible that whilst reconsolidation was not sensitive to NMDA receptor antagonism, it would be prevented with administration of compounds known to prevent protein synthesis.

Inhibition of the protein kinase mTOR can block reconsolidation (Barak *et al.*, 2013; Blundell *et al.*, 2008; Gafford *et al.*, 2011; Hoffman *et al.*, 2015; Lin *et al.*, 2014). Experiment 10 attempted to replicate these findings. Whilst there was no effect of this treatment when animals were tested the day following reactivation, 7 days later a deficit resembling impaired reconsolidation emerged. There were several explanations for this delay-dependent deficit. One possibility was that the results were the result of state-dependent learning.

Administration of protein synthesis inhibitors results in amnesia when given immediately following acquisition of a step-down avoidance memory (e.g. Kameyama *et al.*, 1986). However, recent investigations have demonstrated that pre-test injections of these drugs reverse this deficit (Gisquet-Verrier and Riccio, 2012), with similar results being obtained for an apparent amnesia resulting from post-reactivation injections, or intra-hippocampal infusions of the same compound (Gisquet-Verrier *et al.*, 2015). Lithium chloride (LiCl) treatment, which does not affect protein synthesis (Squire *et al.*, 1975), but does result in gastric malaise resulting in a significant shift in the internal state of the animal, can also render reactivated memories state-dependent (Gisquet-Verrier *et al.*, 2015). These results have been interpreted to suggest that the deficits in memory expression arising from these treatments are the result of a mismatch between the internal state at memory encoding and retrieval. The internal context of the animal at the time of (or just following) memory acquisition becomes a crucial part of the memory trace such that retrieval can only occur when the animal is in a similar state. Similarly, the internal state can become incorporated into a consolidated memory via reconsolidation mechanisms, such that a similar state is required for the memory to be retrieved (Gisquet-Verrier and Riccio, 2012; Gisquet-Verrier *et al.*, 2015; Riccio and Richardson, 1984). It was possible that treatment with rapamycin resulted in the fear memory becoming state-dependent. Because the half-life of rapamycin exceeds 24 hours in the rat (Yatscoff *et al.*, 1995) the internal context may have been sufficiently similar between reactivation and test sessions to permit retrieval 24 hours following drug treatment, but not 8 days later, when the drug had been fully metabolised.

The difference in weight between vehicle and rapamycin treated rats (previously reported in Fifield *et al.*, 2013; Hebert *et al.*, 2014) or some other related factor, may have somehow contributed towards the deficit in freezing reported in drug treated animals. In PR-LTMT1 animals were (likely Hebert *et al.*, 2014) of a similar weight and showed approximately equivalent levels of fear expression. In PR-LTMT2 when the difference in body weight had emerged, as did the deficit in freezing towards the CS in the rapamycin treated animals.

Finally, the decreased freezing in rapamycin treated animals may have been the result of the protein synthesis inhibiting properties of this drug resulting in a deficit in reconsolidation that was not detected 1 day later, perhaps occurring as a result on a reliance of an extended short-term memory systems at this time point.

In a final test, all animals were given pre-retrieval injections of rapamycin. If the memory deficits were occurring as a result of state-dependent amnesia, such a treatment should lead to the loss of the prior amnestic effect of this drug. If the deficits were occurring as a result of the rapamycin induced weight loss, administration of this drug prior to memory retrieval should have no effect, since there was still a significant difference in body weight between the two treatment groups at the time of this test. Similarly, if mTOR inhibition had prevented memory reconsolidation pre-retrieval rapamycin administration should have no effect. As was the case, this treatment resulted in the loss of the amnestic effect of post-reactivation rapamycin, suggesting the prior retrieval deficit was a result state-dependency, although it was possible the deficit was simply the result of passage of time, since a group that did not receive pre-retrieval rapamycin treatment was not included.

Whilst compelling, the state-dependent amnesia explanation cannot account for all deficits occurring through apparent disruptions of reconsolidation. Although few investigations have been conducted to explore this possibility, reductions in contextual fear expression occurring after post-reactivation intra-hippocampal disruption of *Arc* or *zif-268* signalling with oligodeoxynucleotides (ODNs) cannot be restored by pre-retrieval infusion of these compounds (Trent *et al.*, 2015). Whilst Experiment 9 did appear to demonstrate such a state-dependent effect of rapamycin, this drug has not been extensively used in reconsolidation research and the present results cannot be used to refute experiments using different compounds to result in reactivation-dependent amnesia. However, given that it is known that intra-cranial infusions of the widely used protein synthesis inhibitor anisomycin do result in an aversive state (Jonkman and Everitt, 2009; Jonkman and Everitt, 2011) it is a topic worthy of consideration.

Experiment 11 aimed to investigate, under the same conditions as had been used in previous experiments, whether post-reactivation infusion of anisomycin could result in impaired memory expression in a later test. Since used in 2000 to re-ignite the reconsolidation field by Nader *et al.*, intra-BLA infusions of anisomycin have, for better or for worse (Canal *et al.*, 2007; Qi and Gold, 2009), frequently been used to probe whether memory reconsolidation is taking place. Post-reactivation infusions of anisomycin had no effect on memory expression in tests conducted 1 or 8 days after memory reactivation. This suggested that the retrieval parameters used for the majority of experiments in this chapter were not sufficient to engage reconsolidation.

The reasons for the failure to replicate early demonstrations of blockade of fear memory reconsolidation in this chapter were likely two-fold. The NMDA receptor antagonist used was ineffective at preventing consolidation (Experiment 7 & 8) suggesting it may have also been unable to prevent reconsolidation, perhaps due to the recruitment of NMDA receptor independent learning mechanisms. The reactivation conditions used were also insufficient to result in memory reconsolidation (Experiment 11).

Chapter 8: General discussion

Summary of results

The most consistent finding of this thesis was that a memory retrieval session combined with administration of an amnestic agent had no effect on subsequent memory expression. Of the 22 experiments attempting to prevent reconsolidation only in 3 was subsequent memory retrieval impaired as a result of drug administration.

In Chapter 3 the effects of treatment with the N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 during instrumental memory reactivation on subsequent context-induced renewal (CIR) were investigated. Pilot investigations demonstrated that returning animals to a context they had been trained in, following a period of extinction in a second context, resulted in increased responding that was sensitive to devaluation with sensory-specific satiety. This suggested this responding was goal-directed. Treatment with MK-801 prior to a non-reinforced reactivation session interposed between training and extinction did not affect subsequent CIR but did result in an inability to show a selective decrease in responding in response to devaluation of the reinforcer used in training. This raised the possibility there was an impairment in the reconsolidation of the action-outcome (A-O) association underlying the instrumental memory.

Chapter 4 further investigated the potential for disrupting the reconsolidation of A-O memories. Given that pre-reactivation MK-801 treatment had no effect on CIR in Chapter 3, in these experiments animals did not undergo prolonged extinction sessions before testing took place and all the tests took place in the same context. Using the same reactivation parameters as before, treatment with MK-801 had no effect on the expression of A-O associations, now assessed with reinforcer devaluation with lithium chloride (LiCl) induced conditioned taste aversion (CTA) (it was not possible to replicate the devaluation effect previously obtained with sensory specific satiety). In these experiments it appeared the reactivation session led to extinction; NMDA receptor antagonism with

MK-801 in combination with memory retrieval resulted in increased responding at test, suggesting that administration of this compound was preventing extinction. Given that retrieval sessions that result in extinction do not typically lead to reconsolidation (e.g. Flavell and Lee, 2013; Lee *et al.*, 2006b; Merlo *et al.*, 2014) subsequent experiments attempted to determine the retrieval session parameters (conducted in the absence of reinforcer delivery) that would not lead to extinction. However, it was shown that instrumental memories rapidly extinguish and this was true when responding during training was reinforced under variable interval (VI) and fixed ratio (FR) schedules of reinforcement. Whilst the extinction effects reported were fairly modest, there was never any evidence that MK-801 prevented reconsolidation (or that it was taking place) in these sessions; animals treated with this drug responded at similar or increased levels in comparison to vehicle treated controls.

Chapter 5 investigated reconsolidation of pavlovian memories formed during cocaine self-administration. Animals were trained in a way argued to reflect a 'casual-user' model of substance use (e.g. Murray *et al.*, 2012) that has previously been shown to result in the formation of memories amenable to reconsolidation blockade of the conditioned stimulus (CS)-unconditioned stimulus (US) memory (e.g. Lee *et al.*, 2006a) with NMDA receptor antagonism (Milton *et al.*, 2008a). It was not possible to disrupt reconsolidation of these memories, despite attempts to maximise the likelihood of the memory being reactivated with the use of both non-contingent and contingent CS presentation. The failure to prevent reconsolidation with pre-reactivation MK-801 was replicated when the conditioned reinforcing properties of the CS were assessed with its ability to maintain responding in a second-order schedule of cocaine reinforcement. In fact, when assessed with this method treatment with MK-801 resulted in increased responding in comparison to non-reactivated controls, suggestive of a memory strengthening effect. A final experiment in this chapter explored the possibility that the inability to detect an amnestic effect of MK-801 administration was not the result of the reactivation or testing parameters, but that this drug was unable to prevent reconsolidation. To this end, a different NMDA receptor antagonist, CPP, was used. This drug has an increased affinity on GluN2A-containing NMDA receptors (Feng *et al.*, 2004; Feng *et al.*, 2005); studies in fear memories have demonstrated that activation of this subunit, and not GluN2B-containing receptors is required for restabilisation (Milton *et al.*, 2013). Treatment with this drug prior to memory reactivation had no effect on subsequent cue maintained drug-seeking.

The experiments in Chapter 6 attempted to disrupt reconsolidation of responding underlying reward-related memories, likely governed by both pavlovian and instrumental associations. Animals in these experiments had undergone extensive training, likely resulting in the recruitment of

stimulus-response (S-R) habits, governed by distinct psychological and neuroanatomical substrates to that of responding having undergone limited training (Balleine and O'Doherty, 2010; Gasbarri *et al.*, 2014; Giuliano *et al.*, in preparation; Murray *et al.*, 2012; Murray *et al.*, 2015). This was first investigated in animals extensively trained to respond for intravenous cocaine delivery. Treatment with MK-801 prior to a memory reactivation session, designed to maximise prediction error (PE) consisting of both instrumental responding and presentation of the reward-paired stimulus, had no effect on subsequent responding under these conditions. In a second experiment the use of similar training and reactivation protocols, now using a food reinforcer, did appear to result in memory reconsolidation; NMDA receptor antagonism at reactivation decreased food-seeking behaviour the next day. However, this deficit was short-lived; once both the CS and operant response were again paired with reward animals responded at similar levels regardless of drug treatment at reactivation. A final experiment in this chapter demonstrated that repeated reactivation sessions, in conjunction with further drug administration, were unable to ameliorate the fleeting nature of this effect.

In the majority of cases described above it was not possible to disrupt reconsolidation. A final series of experiments explored the possible reasons for these effects, specifically assessing whether they were caused by an inability to prevent this process, and/or whether the retrieval sessions were not resulting in the necessary destabilisation processes required for reconsolidation to occur. Using fear memories as a tool for investigating these possibilities, owing to the short duration and ease at which parametric manipulations can be made in these experiments, a sequence of studies used different memory reactivation procedures and pharmacological tools used to attempt to prevent reconsolidation. There was not any evidence, however, that NMDA receptor antagonism led to blockade of this process, despite manipulation of memory strength, novel contextual information present and extent of PE at reactivation. In fact, MK-801 treatment had no effect on fear memory acquisition, suggesting that this drug might also be unable to prevent reconsolidation, should it be taking place. Following experiments therefore prevented protein synthesis in an attempt to block memory reconsolidation. Whilst pharmacological inhibition of the mammalian target of rapamycin (mTOR) pathway did result in a decrease in subsequent fear expression when given immediately following a memory reactivation session, this did not appear to be reflective of a disrupted reconsolidation, but rather the result of the memory becoming state-dependent. This was indicated by the ability of pre-retrieval administration of rapamycin to reverse the apparent amnestic effect of post-reactivation treatment with this drug (Gisquet-Verrier and Riccio, 2012; Gisquet-Verrier *et al.*, 2015). The ability to update the memory with this information despite treatment with rapamycin suggests that this may have been able to take place via mTOR-independent mechanisms. A final

experiment used intra-basolateral amygdala (BLA) infusions of the protein synthesis inhibitor anisomycin, a frequently used test of whether reconsolidation is taking place (e.g. Ben Mamou *et al.*, 2006; Merlo *et al.*, 2015; Nader *et al.*, 2000), in an attempt to prevent this process. This manipulation was without effect on memory expression. Combined with previous results, this suggested that the reasons for the failure to observe deficits in memory reconsolidation may have been two-fold. Firstly, the amnestic agent (MK-801) used in many of the experiments, in my hands, was not able to prevent consolidation, suggesting this drug may have also been unable to prevent reconsolidation. Secondly, the conditions of retrieval did not appear to be sufficient to result in reconsolidation of pavlovian fear memories.

It is possible that the failure to prevent reconsolidation was not only the result of the use of an inefficacious manipulation to prevent this process, but also that the conditions of retrieval were not sufficient to lead to reactivation of the memory. In many of the experiments the same procedures, equipment and pharmacological agents were the same as previous studies (e.g. Lee *et al.*, 2006a; Lee *et al.*, 2006b; Merlo *et al.*, 2014; Milton *et al.*, 2008a; Nader *et al.*, 2000; Suzuki *et al.*, 2004). Arriving at a conclusion as to why it was not possible disrupt reconsolidation is, therefore, difficult. Some possibilities are explored below, alongside discussion of the cases in which it was apparently possible to prevent reconsolidation and where treatment with MK-801 resulted in increased memory expression. Considerations for future research are also discussed.

NMDA receptor-independent plasticity

The inability to prevent fear memory acquisition with NMDA receptor antagonism raised the possibility that in several of the experiments described in this thesis reconsolidation was taking place, but this process was able to occur in the absence of NMDA receptor activation. This may be surprising, as both memory and postulated plasticity mechanisms (e.g. long-term potentiation (LTP)) are usually demonstrated as being NMDA receptor-dependent. Since its discovery LTP, where repeated stimulation of a synapse results in enduring increases in synaptic strength, has been heralded as a possible neural correlate of learning and memory (Bliss and Lømo, 1973; Bliss and Collingridge, 1993; Malenka, 1994; Stevens, 1998). Following fear memory acquisition, processes similar to LTP take place in the auditory pathways to the lateral amygdala (Rogan *et al.*, 1997) and the shared ability of NMDA receptor antagonists to prevent both LTP (Collingridge and Bliss, 1987) and learning in tasks such as the Morris water maze and (Morris *et al.*, 1986), discrete CS (Kim *et al.*, 1991; Miserendino *et al.*, 1990) and contextual fear (Fanselow, 1994) conditioning has supported

the possible dependence of learning on this process. However, even if LTP is taken as the cellular correlate of learning it is important to acknowledge that under some conditions LTP can still take place without NMDA receptor activation (e.g. Grover and Teyler, 1990; Harris and Cotman, 1986). This NMDA receptor-independent LTP has been shown to take place in the synapses connecting the amygdala and perirhinal cortex (Perugini *et al.*, 2012), hippocampus (Abe *et al.*, 2003) and thalamus (Weisskopf *et al.*, 1999), with at least the latter required for acquisition of discrete CS fear memories (Romanski and LeDoux, 1992).

Like LTP, learning can take place in the face of NMDA receptor antagonism. Whilst initial memory acquisition does appear to depend on these receptors (e.g. Dalton *et al.*, 2012; Fanselow, 1994; Kim *et al.*, 1991; Miserendino *et al.*, 1990; Stiedl *et al.*, 2000), subsequent learning episodes involving similar outcomes can take place independent of their activation. For example, previous experience of solving a Morris water mazes renders learning about subsequent water mazes NMDA receptor independent (Bannerman *et al.*, 1995; Saucier and Cain, 1995) and prior acquisition of contextual fear associations results in later learning episodes of a similar nature being insensitive to NMDA receptor antagonism (Hardt *et al.*, 2009; Sanders and Fanselow, 2003; Wiltgen *et al.*, 2010). Extinction, but not re-extinction of cued fear memories is also NMDA receptor-dependent (Langton and Richardson, 2008; Langton and Richardson, 2010). It appears, therefore, that learning and relearning are mediated by distinct plasticity mechanisms with only the former requiring activation of NMDA receptors. It is not immediately apparent why learning was able to take place in the absence of NMDA receptor activation in the naïve animals used in experiments of this thesis. It is possible that a learning episode was taking place before the experiments began, perhaps during animals' delivery, that meant subsequent acquisition was able to occur without NMDA receptor activation. Little research has been conducted with regard to the similarity between two learning episodes required to render the second NMDA receptor-independent.

The neurochemical substrates underlying reconsolidation of memories acquired via NMDA receptor-independent mechanisms has not been explored. Speculatively, one might posit that if learning has taken place in the absence of activation of these receptors, reconsolidation of these associations might similarly be able to take place in the presence of their antagonists. It was possible that in many cases where attempts were made to prevent reconsolidation this process was occurring independently of NMDA receptor activation; the reactivation conditions described have previously been demonstrated to result in reconsolidation (Lee *et al.*, 2006b; Merlo *et al.*, 2014).

Investigation of this possibility would first require a retrieval session known to result in memory destabilisation, likely indicated by its ability to make a given association susceptible to protein synthesis, but not NMDA receptor activation. Subsequent experiments could then investigate whether reconsolidation of these associations depends similar mechanisms known to underlie NMDA receptor-independent learning, including GluR2-lacking alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activation (Wiltgen *et al.*, 2010) and L-type voltage-dependent calcium channels (Moosmang *et al.*, 2005).

Memory enhancing effects of MK-801 treatment

Several experiments in Chapter 4 showed that MK-801 treatment prior to a reactivation session resulted in an increase in memory expression in comparison to vehicle treated controls. These effects are most likely explained by the ability of this compound to prevent extinction; it is well-established NMDA receptor antagonism can block this process (e.g. Port and Seybold, 1998). Animals in these experiments that did not undergo reactivation sessions also tended to respond more than those that did, consistent with notion that these sessions were resulting in the recruitment of extinction mechanisms. This likely explains why it was not possible to disrupt instrumental memory reconsolidation; reactivation sessions that result in extinction do not typically lead to reconsolidation of the retrieved memory (Flavell and Lee, 2013; Fuchs *et al.*, 2009; Merlo *et al.*, 2014). In Chapter 5 pre-reactivation MK-801 treatment also increased subsequent memory expression, now demonstrated by an enhanced ability of the retrieved CS to support responding in a second-order schedule of reinforcement. Crucially, however, this effect could not be explained by impaired extinction in these animals, since responding was greater than that of non-reactivated controls, instead suggesting a memory enhancing effect of MK-801 treatment.

Remarkably, a similar result of MK-801 to enhance subsequent memory expression was reported for aversive memories; treatment with MK-801 resulted in increased fear expression 8 days after reactivation. Although in this experiment a non-reactivated group was not included, meaning it was possible that NMDA receptor antagonism was preventing extinction, this was highly unlikely as there was no evidence in control animals of a reduction in fear memory expression between reactivation and test. These results raise the possibility that MK-801 was enhancing reconsolidation, as has previously reported to occur as a result of NMDA receptor agonism (Lee *et al.*, 2006b) and protein kinase A (PKA) activation (Tronson *et al.*, 2006). However, NMDA receptor agonism and PKA activation would be expected to result in enhancements in synaptic plasticity, rather than

NMDA receptor antagonism which should prevent it. The capacity of MK-801 to enhance reconsolidation was, therefore, unexpected. That said, there are reports of MK-801 enhancing both consolidation (Gould *et al.*, 2002) and reconsolidation (Flavell, 2015). The most thorough exploration of the memory enhancing effects of NMDA receptor antagonism has come from Honsberger *et al.* (2015), where it was reported that ketamine (also an NMDA receptor antagonist) enhances subsequent memory expression when administered before, but not after memory reactivation. Like the effects of blockade of reconsolidation with anisomycin (Ben Mamou *et al.*, 2006; Milton *et al.*, 2013) this effect was prevented by intra-BLA infusion of the GluN2B receptor antagonist ifenprodil, potentially suggesting the memory enhancing effects of ketamine may rely on destabilisation of the original association and that the increased memory expression occurs through reconsolidation mechanisms (Honsberger *et al.*, 2015). Pre-reactivation ketamine treatments has also been shown to result in enhancements in fear memory reconsolidation in humans (Corlett *et al.*, 2013).

The opposing results of pre-reactivation MK-801 and ketamine administration to result in memory impairments (Lee *et al.*, 2006b; Merlo *et al.*, 2015, Chapter 6) and enhancements (Honsberger *et al.*, 2015), respectively, has been speculatively attributed to a difference in NMDA receptor affinity of these two drugs (Honsberger *et al.*, 2015). It was suggested that compounds with a decreased binding to these receptors (e.g. ketamine, memantine) result in disinhibition of cortical networks, whilst those with a greater activity on NMDA receptors result in their inhibition (Honsberger *et al.*, 2015; Murray *et al.*, 2014). It is not immediately clear, however, how this cortical disinhibition might result in strengthening of a reactivated memory, although it would be predicted that local infusion of NMDA receptor antagonists into the BLA might reconcile some of the memory enhancing effects of these drugs. It is also possible that the memory strengthening effects of MK-801 are due to the non-specific actions of this drug; alongside antagonism of NMDA receptors MK-801 also increases dopamine and serotonin metabolite levels across several regions of the brain (Löscher *et al.*, 1991), increases Fos expression (Sonnenberg *et al.*, 1989), inhibits activity of nicotinic acetylcholine receptors (Amador and Dani, 1991) and increases acetylcholine levels (Hasegawa *et al.*, 1993). It is possible that some of these effects may have contributed towards the memory enhancing ability of administration of MK-801.

Regardless of the pharmacological basis of memory enhancements occurring as a result of MK-801 administration, such increases in memory expression would not be favoured in a therapeutic context. Although MK-801 is not approved for human use, the NMDA receptor antagonists ketamine and memantine are, and both have memory strengthening effects when administered in combination with a memory reactivation session (Honsberger *et al.*, 2015; Samartgis *et al.*, 2012). That

said, both of these compounds have also been reported to have amnestic effects (Alaghband and Marshall, 2013; Duclot *et al.*, 2016); it is clear both from the results of this thesis and those in the literature that the effects of NMDA receptor antagonism are inconsistent and can enhance, reduce or have no impact on memory expression (e.g. Brown *et al.*, 2008; Honsberger *et al.*, 2015; Lee *et al.*, 2006b; Samartgis *et al.*, 2012; Chapters 3, 4, 5, 6 & 7). Although meta-analysis on the ability of NMDA receptor antagonists to prevent reconsolidation of reward-related memories suggests these drugs have a robust effect to do so (Das *et al.*, 2013), taking into account the inconsistent effects of these drugs in this thesis and those reported for fear memory reconsolidation, future research should explore the use of alternative amnestic agents in the prevention of memory reconsolidation.

The particular reason for the inconsistent effects of NMDA receptor antagonism is unclear. One possibility is that age-related changes in availability of NMDA receptors (Armentia and Sah, 2003; Monyer *et al.*, 1994; Sheng *et al.*, 1994) leads to a reconsolidation process that takes place with differing dependence on these receptors throughout a lifespan. Exploration of this possibility would require assessment of the susceptibility of reconsolidation to NMDA receptor antagonism in animals of differing ages, with the view that this will differ between young and old rats. Alternatively, differences in environmental enrichment (EE) may explain the discrepancies reported in works conducted to date. Expression of GluN2A messenger ribonucleic acid (mRNA) is increased in response to EE (Andin *et al.*, 2007), potentially altering the balance between GluN2A and GluN2B signalling, hypothesised to be critical in mediating the amnestic effects of broad-spectrum NMDA receptor antagonism (Milton *et al.*, 2013). Differences between laboratories and development of legislation/policy requiring EE in the of breeding animals may explain the loss of a once robust effects, such as those reported in this thesis. One prediction, therefore, is that exposing animals to differing levels of EE will result in an altered degree of susceptibility of reconsolidation processes to NMDA receptor antagonism.

Highly specific conditions are required for memory reactivation

Whilst the ability of memory acquisition and reconsolidation to occur in the absence of NMDA receptor activation may be able to explain some of the results described in this thesis, in Chapter 7 it was shown that protein synthesis inhibition following retrieval of a discrete auditory CS fear memory also had no effect on the subsequent expression of this association. Given that even NMDA receptor-independent learning likely requires protein synthesis (see Moosmang *et al.*, 2005), this

suggested that the retrieval conditions used were insufficient to result in reconsolidation; the inability of post-retrieval anisomycin administration to reduce subsequent memory expression has previously been attributed to the associations not undergoing reconsolidation in these sessions (Jarome *et al.*, 2015). Whilst the effects of post-retrieval anisomycin were not assessed in pavlovian CS-drug memories formed during self-administration it is possible that the inability to prevent reconsolidation was not due to the independence of NMDA receptor activation in this process, but rather that it was not taking place at all (Blaiss and Janak, 2007; Cammarota *et al.*, 2004; Wang *et al.*, 2009).

Retrieval sessions that do not result in destabilisation and subsequent reconsolidation have previously been reported for all the different types of memories studied in this thesis. Treatment with intra-BLA anisomycin only results in impairments in CIR when combined with a reactivation session that is neither too long or too short in duration (Fuchs *et al.*, 2009). In context-independent instrumental responding it has been reported that only sessions that present the reinforcer in such a way that responses are reinforced in a novel, unpredictable fashion result in reconsolidation of the underlying memories (Exton-McGuinness and Lee, 2015; Exton-McGuinness *et al.*, 2014, see Hernandez and Kelley, 2004; Hernandez *et al.*, 2002). Whilst Brown *et al.* (2008) were not able to disrupt reconsolidation of cue-cocaine associations formed during self-administration, such memories have been shown to undergo reconsolidation (e.g. Lee *et al.*, 2005b; Lee *et al.*, 2006a; Sanchez *et al.*, 2010) that is susceptible to disruptions with the same compound as used in that paper (Milton *et al.*, 2008a). This suggests that the inability to disrupt reconsolidation in the Brown *et al.* study may have been the result of insufficient reactivation conditions, rather than these memories not being able to reconsolidate (although another possibility is that this reconsolidation was taking place independent of NMDA receptor activation). Food paired pavlovian memories are similarly sensitive to the number of CS presentations at reactivation (Flavell and Lee, 2013), alongside the presentation of these cues being contingent upon responding (Lee and Everitt, 2008b). Fear memory reactivation sessions that are too short (Alfei *et al.*, 2015), consist of too many non-reinforced CS presentations (Merlo *et al.*, 2014), do not result in PE (Díaz-Mataix *et al.*, 2013) or do not occur in a novel context (Jarome *et al.*, 2015) have also previously reported to not result in destabilisation of these memories. Strong or old fear memories also appear resistant to reconsolidation (Inda *et al.*, 2011; Milekic and Alberini, 2002; Wang *et al.*, 2005), their destabilisation only occurring in prolonged reactivation sessions consisting of multiple PEs (Suzuki *et al.*, 2004). The repeated finding of this thesis that retrieval sessions do not always result in memory reactivation is, therefore, not novel.

What does seem apparent, however, is that the window for disrupting reconsolidation appears remarkably small, even more so than has been acknowledged to date. Every effort was made to satisfy as many of the possible boundary conditions of reconsolidation. Despite this, in the vast majority of cases it was not possible to trigger memory destabilisation, as indicated by an absence of a reactivation-dependent effect of amnestic agent administration. It is possible that a number of factors acted in tandem to prevent destabilisation from taking place; typically only one parameter was manipulated at a time in an attempt to determine which factor was preventing reconsolidation from occurring. It is possible that a combination of numerous different manipulations of the reactivation sessions may have resulted in memory destabilisation.

If reconsolidation blockade is to offer any promise in the treatment of psychological disorders the reactivation sessions used during these interventions must result in destabilisation of the maladaptive memories. Those arriving in the clinic for the treatment of drug addiction will do so with drug taking histories varying in duration, frequency and patterns of substance abuse. Similarly, the trauma(s) that resulted in the formation of post-traumatic stress disorder (PTSD) will have occurred very differently from one another and taken place months or years prior to seeking treatment. All of these issues will make determining retrieval parameters that result of destabilisation of memories underlying psychological disorders likely the most difficult aspect of development of reconsolidation based treatments for these conditions. In this thesis it was frequently not possible disrupt reconsolidation, despite the tight control over training strength, contingency and memory age that is possible in preclinical research. If memories cannot be destabilised under these conditions it is, perhaps, not all too surprising that several clinical studies have been unable to disrupt reconsolidation resulting in long-lasting reductions in clinical symptomatology (Das *et al.*, 2015a; Saladin *et al.*, 2013; Surís *et al.*, 2013; Wood *et al.*, 2015).

Implications of effects suggesting reconsolidation impairments

Whilst in the majority of experiments it was not possible to disrupt reconsolidation, in some cases treatment with amnestic agents did result in a decrease in subsequent memory expression. These results are discussed below and the wider implications of the nature of these effects addressed.

In Chapter 3 pre-reactivation administration of an NMDA receptor antagonist prior to instrumental memory reactivation subsequently led to a selective impairment in the ability to show a selective decrease in responding in response to devaluation of the reinforcer used in training. This raised the possibility that the retrieval session selectively reactivated the A-O memory, leaving only S-R

associations to govern responding. Whilst there is a wealth of evidence suggesting goal-directed and habitual responding depend on distinct neural circuitries (Balleine and O'Doherty, 2010; Gasbarri *et al.*, 2014) less work has been conducted on the consolidation and reconsolidation of these associations. The fact that treatment with MK-801 impaired the ability of rats to use A-O associations to guide behaviour supports the notion that A-O and S-R memories are governed by discrete neural networks, but also suggests they are updated and reconsolidated separately. However, it was possible that this deficit was not due to a disruption of reconsolidation and was instead a result of treatment with MK-801 alone, rather than administration of this drug in combination with a memory reactivation session. Furthermore, additional inspection of the data raised the possibility that the failure to observe a devaluation effect was not due to an A-O specific memory deficit, but rather a decreased specificity of the pre-feeding treatment. Subsequent experiments using LiCl to induce a CTA, rather than sensory specific satiety, to devalue the reinforcer were unable to demonstrate a specific impairment in the ability of MK-801 treated animals to use the current value of the reinforcer to guide responding. One of the most important criteria for any research, not least related to reconsolidation, is that it is replicable. The inability to do this in this case indicated that the findings of the initial result to prevent expression of goal-directed behaviour should be interpreted with caution.

In Chapter 6 treatment with MK-801 prior to a reactivation session consisting of both instrumental responding and presentation of the reward-paired stimulus led to a decrease in a well-trained food-seeking behaviour. Previous studies exploring similar memories have typically used animals that in which responding is likely goal-directed (Flavell and Lee, 2013; Lee and Everitt, 2008a; Lee and Everitt, 2008b see Belin and Everitt, 2008; Murray *et al.*, 2012). Whilst Monsey *et al.* (2017) report disruption of a cocaine seeking memory in animals having undergone 'extensive' training (24d), the subjects in this experiment were trained on an FR1 schedule, unlikely to result in the recruitment of habitual responding (Murray *et al.*, 2012). Whilst Exton-McGuinness *et al.* (2014) were able to disrupt instrumental responding for a food reinforcer, shown to be habitual, animals had only undergone 10 days of training. Animals in Chapter 6 underwent $\sim$33 training sessions, the longest period of training used in similar studies conducted to date. The ability to disrupt food seeking memories trained under such conditions provides important proof of principle data for the use of such manipulations to treat psychiatric disorders characterised by habitual responding, including drug addiction. However, the decrease in seeking behaviour was transitory and once both the operant response and CS-US pairing were once again reinforced responding was unaffected by prior drug treatment. This is in contrast to deficits occurring as a result of disrupted reconsolidation

of memories underlying drug-seeking that are likely goal-directed, which are maintained despite reminders of the CS-US association (Lee *et al.*, 2006a). The ability of these reinforced sessions to restore apparent disruptions of reconsolidation has previously been used to support the notion these deficits are the result of enhanced extinction, rather than disrupted memory reconsolidation (Fischer *et al.*, 2004; Trent *et al.*, 2015), and may be reflective of a retrieval, rather than storage a deficit (Miller and Springer, 1973). Such effects would not be favoured in a therapeutic context owing to the ease in which expression of the original memory can be recovered following re-exposure to drugs of abuse or trauma. Future reconsolidation studies should ensure that deficits in responding are resistant to such savings effects.

Finally, in Chapter 7 post-reactivation treatment with the mTOR inhibitor rapamycin led to a decrease in fear memory expression when tested 8 days later. Previous studies have reported disruptions of reconsolidation of similar memories with this drug (Blundell *et al.*, 2008; Gafford *et al.*, 2011; Hoffman *et al.*, 2015; Huynh *et al.*, 2014); a decrease in memory expression as a result of this treatment was not a novel finding. However, the fact that this effect was only detected 8 days following initial drug administration suggested this result may have been qualitatively different from those previously reported. Indeed, this deficit was reversed with pre-retrieval administration of the same drug. This led to the possibility that the reactivation session resulted in incorporation of the internal context of drug administration, such that subsequent retrieval was not possible without the same contextual cues present (Gisquet-Verrier and Riccio, 2012; Gisquet-Verrier *et al.*, 2015; Riccio and Richardson, 1984). None of the previous experiments using this compound to prevent reconsolidation have explored whether the results may have been caused by state-dependency. It might be argued that such deficits are not entirely unfavourable in a therapeutic context, given that individuals are unlikely to experience the same interoceptive cues evoked by drug treatment. However, reminders of the association in the absence of these internal cues might result in the memory no longer being state-dependent and the association once again expressed. Whilst state-dependent retrieval deficits can account for some decreases in memory expression occurring as a result of experimentally induced amnesia (Flint Jr. *et al.*, 2013; Gisquet-Verrier and Riccio, 2012; Gisquet-Verrier *et al.*, 2015; Hinderliter *et al.*, 1975; Riccio and Richardson, 1984), not all deficits can be explained as a result of this treatment (e.g. Baratti *et al.*, 2008; Trent *et al.*, 2015). It is also difficult to use this account to explain memory enhancements occurring as a result of administration of drugs including the partial NMDA receptor agonist d-cyloserine (DCS) (Lee *et al.*, 2006b; Lee *et al.*, 2009) or the adrenergic prodrug dipivefrin (Schramm *et al.*, 2016). However, given that it is known that intracranial infusions of the widely used protein synthesis inhibitor anisomycin do result in an aversive

state (Jonkman and Everitt, 2009; Jonkman and Everitt, 2011), future research using this compound should take this possibility into account.

Future research

The most important consideration for future preclinical research will be a more thorough investigation of the conditions under which reconsolidation occurs. Whilst research has been conducted on this topic, the results of this thesis suggest there is still significant progress to be made. Emphasis should be placed on delineation of conditions of retrieval that lead to reactivation that are not so specific they cannot be easily be replicated. Whilst many fear memory reconsolidation studies have conducted the reactivation sessions in novel contexts (e.g. Duvarci and Nader, 2004; Jarome *et al.*, 2012; Nader *et al.*, 2000; Tronson *et al.*, 2006), and Jarome *et al.* (2015) report that this is necessary for destabilisation to occur, Lee *et al.* (2006b) were able to disrupt reconsolidation using a reactivation session in the same context as training. It is possible that in the Jarome *et al.* study reactivation sessions in a familiar context were unable to destabilise the memory because of, for example, the stronger training protocol than that used by Lee *et al.*[1], although this hypothesis was not tested. Future investigations should aim to determine the reactivation conditions that are least susceptible to previously reported boundary conditions of reconsolidation. Such knowledge will be likely be informative in the integration of reconsolidation based treatments for disrupting maladaptive memories in the clinic.

If preclinical research is to provide insight into the conditions under which memories reconsolidate, it is of vital importance that procedures used in these studies appropriately reflect relevant psychiatric disorders. Gaining a detailed understanding of the parameters that permit reconsolidation of a conditioned place preference memory, which has undergone 4 drug-pairings with experimenter administered drug-delivery is unlikely to inform treatments for those suffering from drug addiction, where individuals can undergo an estimated 146,000 drug pairings (Das *et al.*, 2015a) that are under control of the individual. The experiments in Chapter 6 showed that disrupting reconsolidation of a well-established reward-seeking memory resulted in only a short-lasting decrease in responding. Crucially, it only appears this occurs when reconsolidation underlying well-trained appetitive memories is disrupted (Lee *et al.*, 2006a), highlighting the importance of consideration of such factors in preclinical studies. The effects of reconsolidation blockade in drug addicts show a

[1]The Jarome *et al.* (2015) study used 4 shock deliveries paired with the CS, 1mA, lasting 1s, whilst Lee *et al.* (2006b) used half the number of pairings, shock intensity and duration

similar pattern of results; disruption of this process leads to a decrease in cue-induced craving that is lost 1 week after reactivation (Saladin *et al.*, 2013). Future preclinical studies should continue to strive to model aspects of psychological disorders as closely as possible. The results of disrupting reconsolidation of memories that are maximally reflective of psychological disorders will be of most benefit to clinicians in the development of reconsolidation based treatments for these disorders.

Whilst it was possible to disrupt memories formed during extended periods of food-seeking, the same was not true of memories formed during cocaine self-administration. Although it was possible this resistance was unrelated to the extensive training, by virtue of the fact that similar memories formed during short periods of drug taking could also not be disrupted, the prolonged periods of drug-seeking, combined with the increased reinforcing properties of the cocaine US, may have contributed towards this resistance in destabilisation. Given that ensuring memories are destabilised is of utmost importance in developing reconsolidation based treatments future research should also focus its efforts on methods that allow strong memories that are resistant to reconsolidation to destabilise. There is evidence to suggest reactivation sessions conducted multiple times (Robinson and Franklin, 2010), following a delay from training (Wang *et al.*, 2009) and of a longer duration (Suzuki *et al.*, 2004) may make well trained memories more amenable to reconsolidation. However, prolonged reactivation sessions are not always effective at destabilising these associations (Wang *et al.*, 2009; Chapter 7) and repeated reactivations were not able to ameliorate the ephemeral effects of MK-801 treatment in Chapter 6. Another possibility might be to pharmacologically enhance destabilisation. For example, it has been shown that the resistance to reconsolidation that occurs as a result exposure to a stressful situation can be overcome with pre-reactivation DCS administration (Bustos *et al.*, 2010), possibly through activation of GluN2B receptors (Ben Mamou *et al.*, 2006; Milton *et al.*, 2013). Given the importance of disrupting overly intrusive memories that are likely strong by nature further research should be conducted investigating the methods that result in strong memories once again becoming amenable to reconsolidation blockade.

Each of the investigations described above require an amnestic agent that will be effective at preventing reconsolidation. The results of Chapter 7 and others suggest that NMDA receptor antagonists may be of limited use in this regard. One possibility is to probe for molecular markers that reconsolidation is taking place, such as protein ubiquitination (Jarome *et al.*, 2011) or Shank (Lee *et al.*, 2008), *zif-268* (Milton *et al.*, 2008a) or extracellular signal-regulated kinase (ERK) expression (Krawczyk *et al.*, 2016; Merlo *et al.*, in preparation). However, these markers alone are not sufficient evidence that reconsolidation is taking place; this can only be confirmed through disruption of their signalling resulting in a subsequent decrease in memory expression. Attempts to determine

whether reconsolidation is taking place should, therefore, continue to use pharmacological investigations in order to characterise the conditions under which memories become reactivated.

What next for the paradigm of reconsolidation?

The revival of reconsolidation research over the past 15 years resulted in a true paradigm shift in memory research. No longer are memories considered stable indefinitely; it is now acknowledged they can be interfered with and modified following their retrieval, possibly enabling integration of new information into the association. Given the argued importance of maladaptive memories in several psychological conditions, PTSD, phobias and drug addiction being the focus of this thesis, the possibility of targeting reconsolidation mechanisms to lessen the impact of these associations on behaviour and cognition has great potential. Indeed, some progress has been made in this regard; such interventions result in an impressive reduction of fear in spider-phobics (Soeter and Kindt, 2015a) and a remarkable decrease in responses to drug-paired cues can occur as a result of an apparently minor alteration in the manner in which a cue-exposure session is carried out, possibly due to exploitation of reconsolidation mechanisms (Xue *et al.*, 2012). When utilised, it is apparent that these interventions have great potential in the treatment of psychiatric disorders. Unfortunately, the results of this thesis were, on the whole, unable to offer further support for the use of this approach in future clinical interventions, nor was it possible to replicate previously published data from this laboratory and others. Whilst several explanations of these effects have been offered, ultimately, it must be acknowledged that it was not possible to demonstrate conclusive support for the notion that memories can become amenable to disruption following their retrieval. Whilst some factor must have precluded the detection of these effects, likely the result of an independence of memory processes on NMDA receptor activation and/or a subtle factor of the memory reactivation sessions that prevented reconsolidation taking place, it appears that the blockade of this process cannot be depended upon. Perhaps it is time to acknowledge that the fragility of reconsolidation effects warrants exploration of alternative approaches to weaken memories relevant to psychiatric disorders.

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