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Role of D1 Receptor Antagonism in Contextual Fear Learning and Memory

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Thesis submitted to the University of
Nottingham for the degree of Doctor of Philosophy

July 2015

Abstract

Understanding the modulation of contextual fear learning and memory by the neurotransmitter dopamine is important as it could lead to a greater understanding of the mechanisms underlying anxiety disorders. The effect of D1 receptor antagonism during the contextual fear learning and memory stages was investigated. In the first set of experiments the D1 receptor antagonist SCH 23390 (0.1mg/kg; i.p.) was administered systemically before or immediately after contextual fear conditioning to determine whether D1 receptors are involved in the acquisition and/or consolidation stages. This experiment was followed up by investigating the effects of SCH 23390 infusion into the dorsal hippocampus (5µg per side) or amygdala (2.5µg per side) on contextual fear acquisition. The second set of experiments investigated the involvement of systemic SCH 23390 in the reconsolidation, retrieval, destabilization and extinction of contextual fear. SCH 23390 was administered before or immediately after either a short reactivation or longer extinction session. In the destabilization experiment SCH 23390 was administered prior to reactivation and the NMDA receptor antagonist MK-801 (0.1mg/kg; i.p) immediately after to determine if SCH 23390 could rescue the amnesic effects of NMDA receptor antagonism. It was found that systemic and intra-hippocampal but not intra-amygdala SCH 23390 reduced freezing during memory retention testing, twenty four hours and seven days after conditioning. There was no effect of SCH 23390 when immediately given after conditioning. There was also no effect of SCH 23390 when given either before or after reactivation or extinction sessions. The destabilization experiment was inconclusive as MK-801 was not found to impair memory when administered after reactivation. In conclusion, D1 receptors were found to be involved in the acquisition of contextual fear, and this modulation was found to occur in the dorsal hippocampus but not the amygdala. D1 type receptors were not found to be involved in the consolidation, retrieval, reconsolidation or extinction of contextual fear.

Acknowledgements

I would like to thank my parents Susan and Geoffrey for their unwavering support and help.

I would also like to thank all the members of staff at the University of Nottingham who aided me in achieving this PhD; it has been an excellent learning experience.

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Abbreviations

AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
APV	DL-2-amino-5-phosphonovaleric acid
blAMY	basolateral
BLA	basolateral complex of the amygdala
BDNF	brain derived neurotrophic factor
bmAMY	basomedial
BNST	bed nucleus of stria terminalis
8Br-CAMP	Bromoadenosine- 3', 5'- cyclic monophosphate
CA	cornu ammonis division of the hippocampus
CaMKII	calcium/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
cAMY	central nucleus of the amygdala
CFC	contextual fear conditioning
CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
COMT	catechol-O-methyl transferase
CPP	conditioned place preference
CRE	cAMP response element
CS	conditioned stimulus
CREB	cyclic AMP-response element binding protein
DA	dopamine
DARPP-32	dopamine and cAMP regulated phosphoprotein 32kDA
DAT	dopamine transporter
DD	dopamine deficient
DG	dentate gyrus
Dg	diacylglycerol
dHC	dorsal hippocampus
DMP	delayed matching to place
DOPAC	dihydroxyphenylacetic acid
dPAG	dorsal periaqueductal gray
D1R	dopamine receptor 1
D1R KO	dopamine receptor 1 knockout
D2R	dopamine receptor 2
EC	entorhinal cortex
E-LTP	early long term potentiation
fMRI	functional magnetic resonance imaging
FPS	fear potentiated startle
G-protein	GTP-binding protein
IL	infralimbic cortex
InSP ³	inositol 1,4,5-trisphosphate
IP ₃	inositol trisphosphate 3
IPC	intercalated paracapsular islands
laAMY	lateral nucleus of the amygdala
L-LTP	late long term potentiation
LTP	long term potentiation
LTM	long term memory
MAO	monoamine oxidase B
MK-801 maleate	(+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine

mPFC	medial prefrontal cortex
NA	noradrenaline
NBQX	2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione
NMDA	<i>N</i> -methyl <i>D</i> -aspartate
NMDAR	<i>N</i> -methyl <i>D</i> -aspartate receptor
PAG	periaqueductal gray
PCA	Pavlovian conditioned approach
pCREB	phosphorylated CREB
PFC	prefrontal cortex
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase c
PL	prelimbic cortex
PLC	phospholipase C
PRP	plasticity related proteins
PR-LTM	post reactive long term memory
PTSD	post-traumatic stress disorder
SCH 23390	<i>R</i> (+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1 <i>H</i> -3-benzazepine hydrochloride
SN	substantia nigra
STM	short term memory
STP	short term potentiation
TH	tyrosine hydroxylase
US	unconditioned stimulus
vHC	ventral hippocampus
VTA	ventral tegmental area

Chapter One

General Introduction

1.1 Overview

Learning and memory are essential for survival. Acquiring knowledge and being able to store and recall that knowledge to obtain rewards such as food, or to avoid dangers such as predators, could mean the difference between life and death. Learning and memory is therefore a fundamental requirement. In a constantly changing world it is also important that memories can be adapted and modified when new and more relevant information about a situation becomes available. Fear learning and memory can be divided into a number of different stages including acquisition, consolidation and retrieval. Acquisition is when an association or event is originally learnt, consolidation is where this learning is encoded into a stable long term memory representation and retrieval occurs when this memory representation is recalled at a later date. Additional memory stages include reconsolidation and extinction. Reconsolidation is required to return a retrieved, unstable memory back to a stable form. Extinction is where a new memory about an association or event is formed that competes with the original memory. Fear learning and memory is very beneficial, however, if it becomes maladaptive then it can lead to increased stress and development of anxiety disorders. Therefore understanding the basic mechanisms underlying fear learning and memory could lead to insight into such disorders and potentially help in the development of treatments.

Associative learning is where two stimuli are experienced together and therefore become associated, so that if one stimulus is encountered the other stimulus is expected. The Russian physiologist Pavlov was the first person to investigate associative learning (Pavlov, 1927). He discovered that if a bell was rung while a dog was chewing on meat then with time the ringing of the bell alone without the meat was enough to cause the dog to salivate. This indicated that the dog had learnt to associate the ringing of the bell with the delivery of meat. This method is known as classical or Pavlovian conditioning. The bell is the conditioned stimulus (CS), the meat is the unconditioned stimulus (US) and the salivation resulting from the ringing of the bell alone is

the conditioned response. Pavlov also found that if the bell was then subsequently rung numerous times without being followed by the meat, the dogs learnt to no longer associate the two stimuli and stopped salivating to the bell. This is extinction (Pavlov, 1927). Classical conditioning can also be used to investigate fear learning and memory. Auditory fear conditioning is a modification of the method used by Pavlov, where a tone is paired with the delivery of an electrical shock, leading to the expression of fear behaviours in response to the tone alone. Another type of classical conditioning is contextual fear conditioning (CFC; outlined in section 1.2). Contexts are a multisensory set of circumstances that surround an event. For example, a spatial context includes the space and arrangement of objects within the space. Temporal, interoceptive, cognitive and social contexts also make important contributions to an experience (Maren et al., 2013). Contextual fear memory is where a particular context is associated with 'unpleasant' fear inducing stimuli. During CFC a previously neutral context (the CS) is associated with a fear inducing stimulus (the US) so that the context alone induces a fear response. In studies using rats the context is often a fear conditioning chamber and the fear stimulus an electrical footshock (Fanslow and Tighe, 1988). Fear conditioning is an excellent experimental method as it allows control over the different learning and memory stages and memory expression can be easily measured. It has also been demonstrated in a wide array of species from sea snails to humans (Hawkins et al., 1983; Grillon et al., 2006).

Dopamine (DA; outlined in section 1.4) is a neurotransmitter that is widely distributed throughout the brain. To exert its physiological responses dopamine binds to dopamine receptors that are located on neurons within specific brain regions. There are two main families of dopamine receptors, the D1 receptor family and the D2 receptor family. The activation of these receptors by dopamine leads to the initiation of intra-cellular signalling cascades resulting in altered gene and protein expression. Previous research has shown that DA is important in fear learning and memory and the

development of drugs that modulate the effect of DA could potentially be used for pharmacological enhancement of psychological therapy used to treat anxiety. The experiments undertaken in this thesis confirm and extend previous research by investigating the involvement of the D1 receptor signalling in the different contextual fear learning and memory stages.

1.2 Anxiety Disorders and Contextual Fear Conditioning

Brain regions thought to be important for contextual fear learning include the amygdala and hippocampus. The neuroanatomy of these brain regions along with their intrinsic synaptic pathways and some of the numerous lesion and inactivation studies undertaken to ascertain their function during CFC have been outlined in sections 1.2.2 to 1.2.6. The brain pathways thought to underlie CFC have been outlined in section 1.2.7 and human CFC in section 1.2.8. If human CFC becomes maladaptive then increased stress and the development of anxiety disorders such as post traumatic stress disorder (PTSD) can occur. These have been outlined below.

It is potentially beneficial for future therapy advancement to understand the mechanisms and brain regions underlying CFC. Extinction (outlined in section 1.3) based treatments such as exposure therapy, are widely used to treat anxiety disorders. Functional magnetic resonance imaging (fMRI), a non-invasive method where increased blood flow is used as a measure of increased activity in different brain regions of interest, was used in participants with PTSD to assess brain activity during extinction training and recall (Milad et al., 2009). Participants first underwent fear conditioning to a background picture of a room in which a lamp was represented with different coloured lights (e.g. blue, red or yellow). Two of the lamp colours were paired with a shock to the hand and the other was presented without shock. Extinction training was then undertaken in which one of the coloured lamps which was previously associated with receiving a shock was represented in a different background picture and was this time presented without the shock. The next day extinction recall was tested by presenting the original

background pictures and lamp colours. Skin conductance responses were used as a measure of fear. It was found that PTSD patients had impaired extinction recall compared with controls (trauma exposed non-PTSD subjects). It was also found that PTSD patients had higher activation of the amygdala during extinction, whereas lower activation of the hippocampus was found during extinction recall (Milad et al., 2009). One of the main problems with extinction based therapies is that the fear can often return. This is also encountered in experimental extinction studies in the form of spontaneous recovery. The reconsolidation process (outlined in section 1.3), in which the original memory trace can be modulated, is therefore also a potential treatment for anxiety disorders, either alone or in combination with extinction. Spontaneous recovery of fear was found to be alleviated if extinction training was carried out within the reconsolidation time window (Schiller et al., 2010). Extinction training conducted ten minutes, but not six hours, after reactivation was more effective at reducing fear as measured using skin conductance responses (Schiller et al., 2010). This finding however was not replicated in another study (Kindt and Soeter, 2013), although it differed in the use of fear relevant conditioned stimuli (e.g. picture of a spider as opposed to geometric shapes) and behavioural measures (startle responses and expectancy ratings) which could explain the different results obtained (Kindt and Soeter, 2013).

1.2.1 Anxiety Disorders

There are a number of different types of anxiety disorders including simple phobias and PTSD (American Psychiatric Association, 2013). These conditions involve uncontrollable, exaggerated and inappropriate fear responses that impede everyday living and functioning. Simple phobias are where certain things (e.g. snakes or spiders) elicit a strong fear response. Preparedness theory is the idea that fearful things are more likely to be learnt about and remembered because evolution prepared humans and animals for these types of stimuli, due to their potential danger. PTSD is where exceptionally traumatic events have been experienced or witnessed. Incidents of PTSD are

higher in war veterans and people who have suffered physical or sexual assault compared with the general population. An estimated 3% of the UK population will suffer from PTSD at some point in their lives. Traumatic learning underlies anxiety disorders such as phobias and PTSD and therefore some level of fear conditioning is involved. CFC is thought to be particularly relevant in PTSD as the US is unpredictable and background cues that are present during the traumatic experience, when encountered later, are able to trigger the strong emotional responses. For example, in a study examining contextual and cued conditioning in humans, it was found that CFC was stronger in an unpredictable context (Grillon et al., 2006). In this study, the predictable virtual context was a particular room in which the switching on of a virtual lamp with a particular coloured light was paired with receiving hand shocks. In a second virtual room the delivering of shocks was unpredictable and a third 'safe' virtual room was not paired with any shocks. Fear was measured using potentiated startle of the eye-blink reflex to a burst of white noise, behavioural avoidance assessed by which virtual room participants subsequently chose to enter to receive a monetary reward and self reports of subjective feelings of anxiety toward each of the three rooms. It was found that CFC was stronger in the unpredictable context whereas cued fear was stronger in the predictable one (Grillon et al., 2006).

1.2.2 The Neuroanatomy of Contextual Fear

The amygdala and hippocampus (see Fig 1.1) are brain regions that have been shown to have an important role in CFC. The work in this thesis focuses on these structures, which have therefore been outlined in detail below along with studies investigating the input and output pathways to these brain regions, thought to be active during CFC.

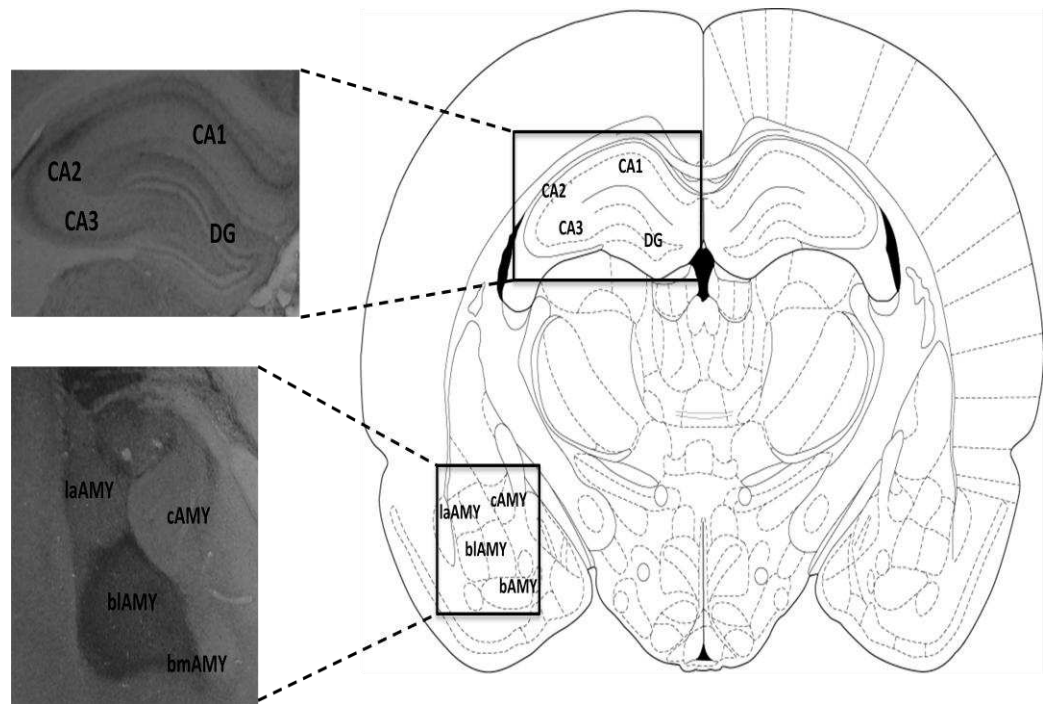


Fig 1.1 Coronal section of the rat brain showing the sub-regions of the hippocampus (DG dentate gyrus, CA1 cornu ammonis 1, CA2 cornu ammonis 2, CA3 cornu ammonis 3) and amygdala (laAMY lateral nucleus, blAMY basolateral nucleus and bmAMY basomedial nucleus, cAMY central nucleus) (modified from Paxinos and Watson, 2009)

1.2.3 The Amygdaloid Complex

The amygdaloid complex is located in the medial temporal lobe (see Fig 1.1). It comprises around thirteen nuclei including the lateral (laAMY), basolateral (blAMY), basomedial (bmAMY; also known as the accessory basal), central (cAMY), cortical and medial nuclei as well as the lateral and ventral intercalated paracapsular islands (IPC) and the amygdala part of the bed nucleus of stria terminalis. The laAMY, blAMY and bmAMY can be grouped together as the basolateral complex (BLA). The amygdala nuclei can be further subdivided for example the blAMY and bmAMY can each be divided into magnocellular and parvocellular subdivisions and the cAMY includes the lateral and medial subregions (Sah et al., 2003; Lee et al., 2013).

Information in the amygdaloid complex flows medially from the BLA, the main input structure, to the medial cAMY, the main output structure (see Fig 1.2). Unimodal and polymodal sensory information enters the amygdala via the laAMY or bmAMY. The laAMY projects to and receives reciprocal projections from the bmAMY and blAMY. The blAMY and bmAMY both project to the medial division of the cAMY. The medial cAMY receives inputs from many amygdala nuclei but only sends sparse reciprocal projections (Sah et al., 2003). Information is trafficked between the BLA and cAMY via the IPCs. The BLA, cAMY and IPCs are composed of different neuronal types. The BLA contains mainly pyramidal-like glutamergic projection neurons, whereas cAMY contains medium spiny GABAergic projection neurons. The IPCs are composed of GABAergic interneurons (Sah et al., 2003). Under normal, non-fearful conditions the BLA glutamergic projection neurons are kept under basal suppression by the GABAergic inhibitory neurons located in the laAMY and lateral IPC. Likewise, the medial cAMY GABAergic projection neurons are inhibited by GABAergic neurons located in the lateral cAMY and ventral IPC (Lee et al., 2013).

1.2.4 The Amygdaloid Complex and Contextual Fear Memory

Lesion studies of the amygdala demonstrate that it is important for contextual fear memory. Electrolytic lesions of the amygdala created by passing anodal current prior to CFC impaired freezing behaviour (a commonly used measure of fear, see section 1.2.7) immediately and twenty four hours later (Kim et al., 1993; Phillips and LeDoux, 1992). Excitotoxic BLA lesions using N-methyl D-aspartate (NMDA) prior to or after conditioning impaired freezing to background context indicating that the BLA lesions result in both anterograde and retrograde amnesia (Maren et al., 1996a). Inactivating the BLA temporally, using muscimol (a GABA_A receptor agonist) prior to CFC also impaired freezing twenty four hours later (Helmstetter and Bellgowan 1994; Muller et al., 1997; Maren and Holt, 2004). However other studies found that rats with lesions of the BLA can learn contextual fear with extensive over-training (Maren, 1999; Ponnusamy et al., 2007). Over-training on the other

hand, did not prevent retrograde amnesia (Maren, 1999; Ponnusamy et al., 2007). This implies that the BLA is normally required for the acquisition of contextual fear; however, if the BLA is inactivated rats can learn but do so inefficiently. This would indicate that other brain structures are able to compensate for the loss of the BLA. The bed nucleus of the stria terminalis (BNST) has been shown to be able to do this as lesions of both the BLA and BNST prevented acquisition of contextual fear after extensive over-training, whereas BLA lesions alone did not (Poulos et al., 2010). As mentioned above, another crucial structure for CFC is the hippocampus, which is discussed in more detail below.

1.2.5 The Hippocampal Formation

The hippocampal formation (see Fig 1.1) is located in the medial temporal lobe and includes the hippocampus proper, dentate gyrus (DG), subiculum, presubiculum, parasubiculum and entorhinal cortex (EC) (Golgi et al., 2001). The three sub regions of the hippocampus proper are named cornu ammonis (CA1, CA2 and CA3). The hippocampal formation can be divided into dorsal, intermediate and ventral regions (Fanselow and Dong, 2010). The dorsal hippocampus (dHC) is thought to be predominantly associated with cognitive and spatial processes whereas the ventral hippocampus (vHC) with its direct projections to the amygdala is thought to be important for emotions such as fear (Fanselow and Dong, 2010).

Information flows through the hippocampal formation via a unidirectional intrinsic pathway (see Fig 1.2). Polymodal sensory information from the cortex (e.g. the perirhinal and postrhinal cortices) and subcortical (e.g. the thalamus) regions enters the superficial layer of the EC (Anderson et al., 2007). The EC projects to the DG via the perforant pathway. The DG granule cells extend their mossy fibres to the CA3 region which, in turn, projects to the CA1 via the Schaffer collaterals. The CA1 projects to both the subiculum and the deep layers of the EC. The subiculum also projects to the EC along with the presubiculum and parasubiculum. The presubiculum and

parasubiculum send projections to subcortical regions whereas the EC projects back to the cortex, closing the pathway loop (Anderson et al., 2007). The principal neuronal type of the hippocampus proper, subiculum, presubiculum, parasubiculum and EC are glutamatergic pyramidal neurons, whereas the principal cells in the DG are the granule cells. All regions of the hippocampal formation contain many different types of inhibitory GABAergic interneurons (Anderson et al., 2007).

1.2.6 The Hippocampal Formation and Contextual Fear Memory

Numerous studies have been undertaken in which permanent or transient lesions of the hippocampus have been used to assess its involvement in contextual fear. These studies produced mixed findings however on balance it would seem that the dHC and most likely the vHC are important in contextual fear under normal circumstances. Initial studies found that lesions of the dorsal or ventral hippocampus caused anterograde amnesia of contextual fear (Kim et al., 1993; Young et al., 1994). Electrolytic (using anodal current) and neurotoxic (using NMDA) dHC lesions prior to conditioning impaired freezing twenty four hours later (Kim et al., 1993; Young et al., 1994). Temporary dHC or vHC inactivation using muscimol was also found to impair CFC (Zhang et al., 2014). Likewise, inactivation of the vHC using tetrodotoxin (Na^+ channel inhibitor) or muscimol blocked CFC (Bast et al., 2001; Zhang et al., 2014). Other studies, however, found that while dHC neurotoxic lesions (using NMDA) made after CFC produced severe retrograde amnesia, lesions made prior to conditioning did not (Maren et al., 1997). Muscimol infusions into either the dHC or the vHC prior to CFC were also not found to impair acquisition (Matus-Amat et al., 2004; Maren and Holt, 2004). Lesioned rats did not learn as efficiently as control rats but this was overcome with increased conditioning trials, whereas lesions made after conditioning resulted in complete retrograde amnesia (Wiltgen et al., 2006). It would therefore seem that the training protocol strongly influences whether or not hippocampal lesions prior to conditioning produce anterograde amnesia. Less challenging CFC protocols (i.e. with greater number of shocks and context

exposure) would appear to be able to be acquired without the hippocampus (Fanselow, 2010). The dynamic memory systems theory postulates that under normal circumstances the hippocampus is important in contextual fear acquisition, however if the hippocampus is unavailable, other competing memory systems can compensate for its loss (Fanselow, 2010). The infralimbic cortex (IL) and prelimbic cortex (PL) subregions of the medial prefrontal cortex (mPFC) have been proposed to be a competing memory system for contextual fear as dHc and IL or PL NMDA neurotoxic lesions prior to CFC resulted in impaired learning whereas dHc lesions alone did not (Zelikowsky et al., 2013).

1.2.7 Fear Conditioning Pathways

During CFC, contextual information inputs to the amygdala from the hippocampus. Somatosensory pain information from the spinothalamic tract terminates in the posterior thalamus which in turn projects to the amygdala. The amygdala has therefore been proposed to be a potential site of CS-US convergence during CFC (Fendt and Fanselow, 1999; LeDoux, 2000). The amygdala connects to brainstem regions involved in eliciting physiological responses and behavioural expression of fear (e.g. freezing behaviour, release of glucocorticoids; Fendt and Fanselow, 1999; LeDoux, 2000). This is discussed in more detail below.

Input pathways:

The amygdaloid complex receives both modality specific and polymodal sensory inputs and is therefore proposed to be the site of the CS-US association (LeDoux, 2000). Polymodal inputs project to the amygdala from the hippocampal formation and prefrontal cortex (Sah et al., 2003). Using the anterograde tracer *Phaseolus vulgaris*-leucoagglutinin (which is taken up by the cell bodies and transported by the axons to the synapses), extensive reciprocal connections between the ventral hippocampal formation and amygdaloid complex have been identified (Canteras and Swanson, 1992; Pikkarainen et al., 1999). Heavy reciprocal projections exist between the BLA

and the EC which also projects to the cAMY but does not receive reciprocal projections from this amygdala subregion (Pikkarainen et al., 1999). The blAMY and bmAMY project to CA3, CA2 and CA1 but these are only reciprocated by the CA1. The subiculum projects to the BLA and cAMY and receives projections back from the blAMY and bmAMY (Pikkarainen et al., 1999). Hippocampal nuclei such as the DG that do not have direct connections with the amygdala can receive information via intra-hippocampal circuitry (Pitkanen et al., 2000). In another study a recombinant virus that expressed channel rhodopsin (a protein that can be activated by light) fused with the fluorescent markers mCherry or eYFP was injected into the vHC. Dense fluorescent labelling was observed in the blAMY (Hubner et al., 2014). Both principal neurons and interneurons in the blAMY were shown to receive excitatory glutamatergic monosynaptic projections from the vHC (Hubner et al., 2014). It has also been shown that high frequency electrical stimulation of the ventral angular bundle which projects between the hippocampus and BLA produced long term potentiation (LTP) in the BLA of anesthetized rats (Maren and Fanselow, 1995). Likewise high frequency electrical stimulation in the BLA was found to produce LTP in the DG of the hippocampus (Abe et al., 2003). There are also extensive connections between the amygdala and the mPFC which includes the PL and IL regions. The PL sends dense projections to the blAMY and cAMY and is thought to be important for fear expression whereas the IL projects to a wider distribution of amygdala nuclei including the BLA, cAMY and IPCs and is proposed to suppress fear (Vertes, 2004; Hubner et al., 2014).

Modality specific nociceptive information from the spinothalamic tract has been shown to input to the amygdala via the posterior thalamus. Spinothalamic tract neurons have been shown to terminate in the posterior thalamus. The anterograde tracer wheat germ agglutinin-conjugated horseradish peroxidase was injected into the spinal cord and found to terminate in the posterior thalamus which includes the medial geniculate and the posterior intralaminar nucleus (LeDoux et al., 1987). Lesions of the

posterior thalamus were found to block the acquisition of auditory fear conditioning (LeDoux et al. 1986). Projections from the posterior thalamus, including the medial geniculate body and the posterior intra laminar nucleus have been shown to terminate in a number of amygdala nuclei including the laAMY, blAMY, bmAMY and cAMY (LeDoux et al. 1985; LeDoux et al., 1990). During fear conditioning local inhibitory circuits within the amygdala are thought to control the flow of information between the BLA and cAMY enabling activation of output pathways and expression of fear (see Fig 1.2). Cortical projections (most likely from the mPFC) suppress feedforward inhibition of the GABAergic neurons located in the lateral IPC thereby disinhibiting the glutamatergic projection neurons of the BLA. The bmAMY and blAMY project to the medial cAMY leading to activation of GABAergic output neurons projecting to the periaqueductal grey (PAG) (Lee et al., 2013) discussed in more detail below.

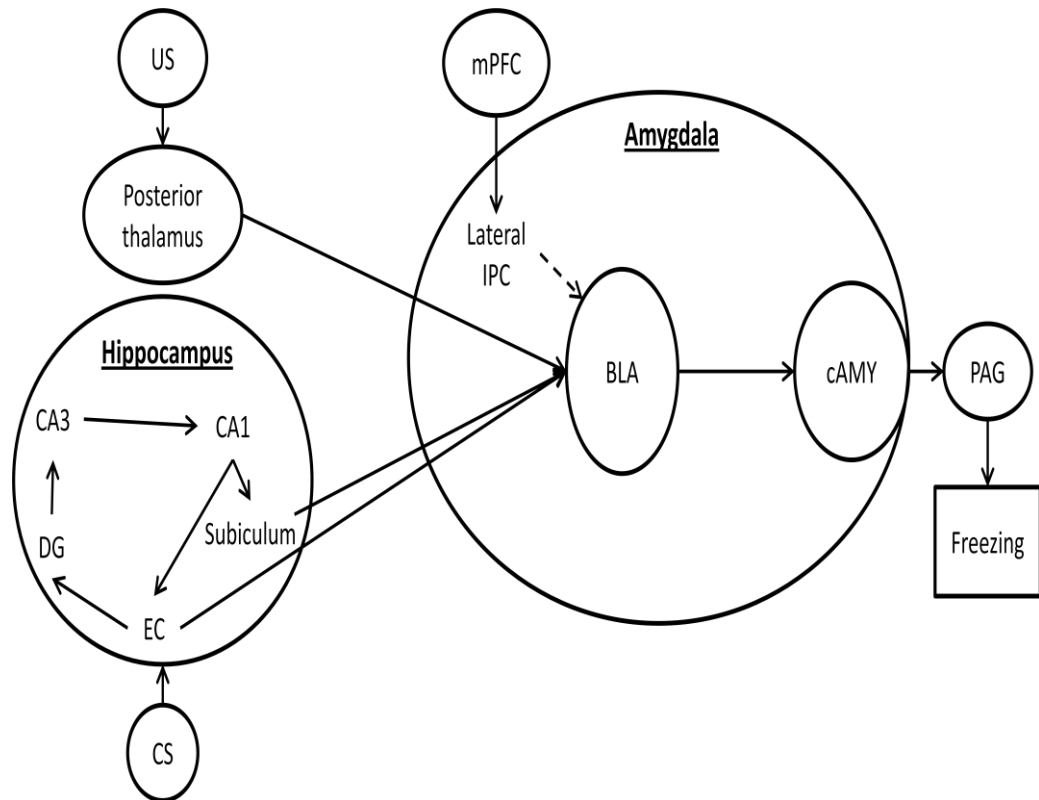


Fig 1.2 Contextual fear conditioning circuitry

During CFC US information inputs to the BLA via the posterior thalamus and contextual CS information inputs to the BLA from the EC and subiculum. The inhibition of the BLA by the lateral IPC is disinhibited by projections from the mPFC. The BLA projects to the cAMY which in turn projects to the PAG leading to the expression of freezing behaviour.

Output pathways:

The medial CE is thought to be the main output region of the amygdaloid complex and therefore has an important role in fear expression (LeDoux, 2000). The main behavioural measures of fear expression include freezing behaviour (see below), release of corticosteroids, hypoalgesia (pain reduction), increased arterial pressure and heart rate and suppression of appetitive responding. Lesions of the corticomедial region of the rat amygdala, which includes the cAMY, resulted in reduced freezing responses to a natural predator (the cat) and to placement in a context previously associated with footshocks (Blanchard and Blanchard, 1972). Lesions of the cAMY and BLA prior to CFC impaired conditional hypoalgesia and freezing

twenty four hours later (Helmstetter, 1992) and arterial pressure and heart rate were increased in rats following electrical stimulation of the cAMY (Iwata et al., 1987).

Freezing behaviour is defined as no movement apart from that caused by respiration. It is an adaptive fear response of rats as it makes them less likely to be observed and therefore attacked by predators. Experimentally, freezing behaviour can be easily measured and is the method used in the experiments carried out in this thesis. The assessment of freezing behaviour assumes that if a rat is moving (i.e. not freezing) then it is not afraid. However some rats will express their fear in a more active form (i.e. try to escape the chamber). Activation of cAMY type 1 cells, which display a prominent depolarizing after potential, results in suppression of cholinergic ventral forebrain neurons that are important for exploratory behaviour leading to passive expression of fear (e.g. freezing) whereas inhibition of these neurons leads to active fear responses (Gozzi et al., 2010). Differences in the numbers of cAMY type 1 cells could therefore potentially explain variation in freezing levels seen between rats given the same conditioning training (Gozzi et al., 2010).

The cAMY projects to brainstem regions important for freezing behaviour for example the PAG. The PAG has been shown to be required for freezing behaviour as lesions impaired freezing to footshock administration or the presence of a cat (Liebman et al., 1970; LeDoux et al., 1988; De Oca et al., 1998) and stimulation of the dorsal PAG (dPAG) results in freezing and escape defensive behaviours in rats (Brandao et al., 1982). The PAG is also important in suppression of pain; ventral and dorsal lesions impaired hypoalgesia (as assessed by tail flick; Helmstetter and Tershner, 1994). PAG lesions do not however, affect arterial pressure (LeDoux et al., 1988; Helmstetter and Tershner, 1994), whereas lateral hypothalamus lesions do (LeDoux et al., 1988). The hippocampus and amygdala have been shown to be involved in PAG dependent freezing behaviour. Lesions of the vHC but not dHC increased the threshold of dPAG stimulation that elicited freezing or escape responses

but did not impair freezing behaviour following dPAG stimulation (Ballesteros et al., 2014). However, muscimol inactivation of the laAMY, BLA or cAMY had no effect on the freezing and escape thresholds but did impair post stimulation freezing (Martinez et al., 2006).

1.2.8 Contextual Fear Conditioning in Humans

A number of studies have been undertaken to investigate the involvement of the amygdala and hippocampus during CFC in humans. These studies have yielded mixed results. In one study, sustained contextual anxiety was found to increase cerebral blood flow in a number of regions including the hippocampus, PFC and PAG but not the amygdala. In contrast, visual cues predicting shocks resulted in increased cerebral blood flow in brain regions including the PFC and amygdala but not the hippocampus (Hasler et al., 2007). The effect of CFC on hippocampal and amygdala volume was investigated in another study (Pohlack et al., 2012). The conditioned contextual stimuli (CS^+ and CS^-) were two coloured backgrounds (orange and blue). CS^+ was paired with an electrical shock to the right thumb whereas CS^- was unpaired. It was found that participants with larger hippocampal volume were more efficient at acquiring contextual fear compared to those with smaller volumes and that the posterior hippocampus (equivalent to the dHC in rats) was most important for this. Amygdala volume, however, was not found to affect CFC (Pohlack et al., 2012). In another study using the same conditioning methods it was found that there was increased activity in the hippocampus and anterior cingulate cortex during early acquisition to the CS^+ whereas the amygdala and inferior frontal cortex (part of the PFC) was found to be active during late acquisition. Connectivity analysis showed correlated activity between the hippocampus and anterior cingulate cortex during acquisition (Lang et al., 2009). Other studies used pictures of rooms as contextual stimuli. One study found that the hippocampus but not the amygdala was activated by CS^+ presentation (Marschner et al., 2008). However in another study the amygdala was found to be activated by the CS^+ (LaBar et al., 1998). Both the hippocampus and the amygdala showed increased activity in a study in which

virtual reality apparatus was used to create a more realistic context (Alvarez et al., 2008). On balance the above studies indicate that the human hippocampus and amygdala, similar to that found for the rat, are involved in contextual fear memory processing.

1.3 Stages of Learning and Memory

Learning and memory can be divided into a number of different stages; these include acquisition, consolidation, retrieval, reconsolidation, destabilization and extinction. Learning or acquisition is the first stage in memory formation. In CFC this is where the animals learn to associate the fear conditioning chamber with the footshocks. Memory acquisition has been proposed to involve Hebbian mechanisms. The induction of LTP fits with the Hebbian model of learning and is thought to underlie memory acquisition. Consolidation is where a short term memory (STM), which is unstable and vulnerable to manipulation, is converted to stable long term memory (LTM). This idea was first proposed in 1900 by Muller and Pilzecker who found, working with humans, that interference of new learning impaired original learning (McGaugh, 2000). Further evidence supporting this theory came initially from rodent studies showing that electroconvulsive shock, if applied directly after memory acquisition, disrupted memory formation leading to retrograde amnesia. In contrast the central nervous system stimulant strychnine (which acts by blocking glycine receptors) led to memory enhancement (Duncan, 1949; McGaugh and Krivanek, 1970).

Retrieval or recall is the activation of a previously consolidated memory trace. In fear conditioning, a CS-US association can be retrieved by presentation of the CS alone. It has been shown using transgenic mice that some of the same amygdala neurons that were activated during auditory fear conditioning were also active during retrieval (Reijmers et al., 2007). Expression refers to the behaviours that are associated with the memory when it is retrieved; for example, freezing is a classic fear behaviour that is expressed in mice and rats. Experimentally it can be difficult to distinguish between retrieval and expression. Many experiments use expression (i.e. freezing behaviour) as a measure of retrieval and the terms are often used interchangeably. The term retrieval has been used in this thesis, although in most of the studies outlined it cannot be ruled out that expression, rather than retrieval, is being affected.

Retrieval and expression are independent processes and therefore caution should be taken when interpreting results (Barreiro et al., 2013).

Reconsolidation is where a destabilized memory is returned to a stable form. It has been shown by numerous studies that if a consolidated memory is reactivated it can become labile and vulnerable to manipulation and therefore requires reconsolidation in order to be restabilized again. For example, electroconvulsive shock applied after reactivation of a consolidated memory impairs that memory (Misanin et al., 1968), whereas strychnine application causes enhancement (Gordon, 1977). This provided support for the proposal that memories either newly acquired or already consolidated should be classed as either being in an inactive state in which they are relatively stable or an active state in which they are vulnerable to manipulation (Lewis, 1979). The process of reconsolidation has been shown across a large number of memory paradigms and species, including humans (Walker et al., 2003). Reconsolidation has been shown to depend on many of the same but also some different macromolecules as consolidation. For example, it was found using antisense oligodeoxynucleotides (which inhibit local protein synthesis) infused into the DH that the immediate early gene *Zif268* and brain derived neurotrophic factor (BDNF) show doubly dissociable roles in reconsolidation and consolidation, respectively, of contextual fear memory (Lee et al., 2004). In rats infused with BDNF antisense oligodeoxynucleotides, consolidation of LTM was impaired, whereas *Zif268* antisense oligodeoxynucleotides had no effect. *Zif268* antisense oligodeoxynucleotides infused rats, on the other hand, had impaired post reactive long term memory (PR-LTM; memory tested following a reactivation session) whereas BDNF antisense oligodeoxynucleotides infused rats did not (Lee et al., 2004). Reconsolidation has been shown to be a separate process from extinction (Duvarci et al., 2006). In this study each rat was trained to have two different auditory fear memories by using two different CSs (a tone or white noise). The next day one of the auditory memories was reactivated for a short time period to induce reconsolidation whereas the other auditory memory was reactivated

for a longer period to induce extinction. Anisomycin was then infused into the BLA. It was found that reconsolidation but not extinction was impaired indicating that the two processes are independent (Duvarci et al., 2006). The process of reconsolidation has been extensively studied; however, in order for a memory to be restabilized it must first undergo a destabilization process. Recent studies have started to investigate this memory stage.

Extinction is where repeated or prolonged presentation of an unreinforced CS results in a new memory being formed that competes with the previously learnt association to suppress conditioned behaviour (Pavlov, 1927). For example, previously conditioned contextual stimuli presented for prolonged durations without footshocks are no longer feared, as measured by reductions in freezing behaviour. Extinction is new learning and includes a number of stages, including acquisition, consolidation and retrieval (Quirk and Mueller, 2008). More recently it has been demonstrated that extinction memories can also undergo reconsolidation (Rossato et al., 2010). Following extinction training, the original conditioned response can spontaneously recover with time. Conditioned responses have also been found to be reinstated if the animal is exposed to the original unconditioned stimulus or renewed if the conditioned stimulus is presented in a different context to where extinction training occurred (Herry et al., 2010). Because fear memories can undergo spontaneous recovery, renewal and reinstatement after extinction it has generally been accepted that extinction involves new learning that suppresses, rather than erases, the fear memory.

Numerous neurobiological mechanisms have been demonstrated to be required during the different learning and memory stages. These include LTP induction or reversal, protein synthesis or degradation, *N*-methyl *D*-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) ionotropic glutamate receptor activation and synthesis of macromolecules such as cyclic AMP-response element binding protein (CREB). Studies investigating the involvement of these mechanisms during acquisition,

consolidation, retrieval, reconsolidation, destabilization and extinction have therefore been outlined below.

1.3.1 The Neurobiology of Acquisition

Research has demonstrated that LTP induction and NMDA receptor (NMDAR) and AMPA receptor (AMPA) activation are involved in the process of memory acquisition. These studies have been outlined below.

1.3.1.1 Acquisition and Long Term Potentiation

In his influential book 'Organization of Behaviour' published in 1949, Hebb proposed that increased synaptic efficiency could be brought about by concurrent activation of pre- and post-synaptic neurons (Sejnowski, 1999). A type of activity-dependent plasticity is LTP which is a lasting enhancement in synaptic efficiency in a post-synaptic neuron brought about by stimulation of both neurons and is a form of Hebbian plasticity. LTP has been demonstrated in both the hippocampus (Bliss and Lomo, 1973) and amygdala (Rogan et al., 1997) and has been produced in the DG by high frequency stimulation of the BLA (Abe et al., 2003). It has been shown that LTP is induced in the hippocampus of rats by fear learning (Whitlock et al., 2006). Theta (4-12 Hz) and gamma (30-100 Hz) oscillations precede electrically-induced LTP in the hippocampus of awake-behaving rats (Bikbaev and Manahan-Vaughan, 2008).

1.3.1.2 Acquisition and NMDA Receptors

LTP can be induced by activation of NMDARs (Malenka and Nicoll, 1999). NMDARs are ionotropic receptors that require binding of glutamate and sufficient depolarization leading to movement of Mg^{2+} , opening up the pore and enabling the influx of cations, including Ca^{2+} . Because of this unique property of NMDARs they have been proposed to act as coincidence detectors and fit well with Hebb's model of learning (Sejnowski, 1999). NMDARs are heterotetramers composed of GluN1 and GluN2 subunits. Activation of NMDARs enables the influx of cations, including Ca^{2+} , into the dendrite which is a critical trigger of LTP, leading to autophosphorylation of calcium-

calmodulin-dependent protein kinase II (CaMKII); this process plays an important role in memory formation (Silva, 2003). Studies using a variety of NMDAR antagonists have demonstrated that they are required during fear learning. In one study, systemic administration of the non selective NMDAR antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) prior to conditioning impaired freezing to contextual, but not auditory stimuli twenty four hours later (Gould et al., 2002). This effect was found to be mediated by the GluN2B subunit of the NMDAR as systemic administration of ifenprodil (GluN2B antagonist) impaired the acquisition of both auditory fear conditioning and CFC (Rodrigues et al., 2001). Intraventricular infusion of another NMDAR antagonist DL-2-amino-5-phosphonovaleric acid (APV) has also been shown to impair the acquisition of CFC (Kim et al., 1991). Studies in which the NMDAR antagonists were infused into the amygdala and hippocampus have shown that these brain regions are important in NMDAR mediated memory acquisition. Intra-amygdala infusion of APV blocked acquisition of second order fear conditioning (Gewirtz and Davis, 1997) and fear potentiated startle (FPS) (Miserendino et al., 1990). Second order fear conditioning involves two conditioned stimuli; for example, two distinct tones, one (CS1) of which is associated with a footshock so that it elicits a fear response. CS1 is then associated with CS2 so that it then also elicits a fear response when presented alone. FPS is where a salient stimulus, such as a footshock, is associated with a neutral stimulus, such as a light; the effect of then presenting the light alone with a startling noise is measured. If an association between the shock and light has been learnt, then the light when presented with the noise will potentiate the startle reflex. Intra-amygdala APV has also been shown to block the acquisition of contextual fear (Maren et al., 1996b) and this was shown to involve the GluN2B subunit (Rodrigues et al., 2001). Intra-hippocampal APV infusions were found to impair CFC (Young et al., 1994; Quinn et al., 2005). Intra-hippocampal infusions of MK-801 also blocked contextual but not auditory cued fear (Bast et al., 2003). In another study however, it was found that GluN2A-containing, rather than GluN2B containing, NMDARs are important for memory

acquisition (Dalton et al., 2012). Systemic administration of NVP-AAM077 (a selective GluN2A antagonist), but not Ro25-6981 (a GluN2B antagonist), prior to conditioning was found to impair auditory fear acquisition (Dalton et al., 2012). It is also important to point out that not all LTP is dependent on NMDAR. For example, LTP induction in the laAMY has been shown to be dependent on either voltage gated Ca^{2+} channels or NMDARs depending on the stimulation protocol used, with both likely contributing to memory formation *in vivo* (Bauer et al., 2002). Moreover, LTP in the BLA-DG pathway was found to be NMDAR independent (Abe et al., 2003).

1.3.1.3 Acquisition and AMPA Receptors

The AMPAR is a heteromer composed of various combinations of GluR1, GluR2, GluR3 and GluR4 subunits (Mayer, 2005). During LTP induction AMPARs are phosphorylated by CaMKII. This increases their channel conductance and boosts the numbers of GluR1 containing AMPARs trafficked to the dendrite spines (Malenka and Nicoll, 1999; Shi, 1999). Increased GluR1-containing AMPARs were found in the dendritic spines of amygdala neurons following auditory fear conditioning (Rumpel et al., 2005; Nedeleescu et al., 2010). Synaptic delivery of GluR1 containing AMPAR was also found to be required in the dHC for inhibitory avoidance acquisition (Mitsushima et al., 2011). Inhibitory avoidance involves the animal being initially placed in a safe, light compartment of the training apparatus. When it first moves into the dark compartment a door shuts, preventing the animal from re-entering the safe area. When the animal reaches the far end of the dark compartment it receives a footshock. The animal's memory is later tested by placing it back into the chamber and measuring its latency to enter the dark compartment. Mice in which the GluR1 subunit of the AMPAR had been knocked out showed an absence of LTP in the amygdala and impaired acquisition of contextual and auditory fear (Humeau et al., 2007). Spatial working memory was also impaired in mice lacking the GluR1 subunit (Reisel et al., 2002).

1.3.2 The Neurobiology of Consolidation

Studies have shown that memory consolidation requires protein synthesis and activation of different molecular pathways, leading to transcription of genes important for plasticity (e.g. changes to dendritic structure). Synaptic tagging and capture is a theory for how early LTP (E-LTP) is converted to late LTP (L-LTP), a process that could explain memory consolidation at the cellular level and has therefore been described in more detail below.

1.3.2.1 Consolidation Requires Protein Synthesis

Much of what is known about memory today was initiated by studies undertaken on a simple form of aversive learning in the marine snail *Aplysia*. Work carried out by Kandel and colleagues showed that the gill withdrawal reflex (a defensive reflex where stimulation of the siphon causes withdrawal of the gill) was enhanced when preceded by shocks to the tail (Hawkins et al., 1983). By reconstituting this reflex in cell culture they found that application of the neurotransmitter serotonin simulated the effect of tail shock with one application, producing short-term facilitation that lasted a few minutes, whereas five applications of serotonin induced long-term facilitation that lasted over twenty four hours. Long-term, but not short-term, facilitation was found to require both protein and RNA synthesis as anisomycin and emetine (protein synthesis inhibitors), and actinomycin and α -amanitin (RNA synthesis inhibitors), blocked the actions of five applications of serotonin but not one (Montarolo et al., 1986). This work was subsequently followed up with work in other animals including mice and rats. It was found that mice administered anisomycin (a protein synthesis inhibitor) after auditory fear conditioning displayed reduced freezing to both context and cued stimuli twenty four hours later (Bourtchouladze et al., 1998). In another study anisomycin infusion into the rat BLA immediately after auditory fear conditioning impaired LTM but not STM (Schafe and LeDoux, 2000). Anisomycin infused after six hours however did not affect LTM (Schafe and LeDoux, 2000). The mRNA synthesis inhibitor actinomycin-D was infused into the BLA prior to auditory fear conditioning. Freezing to both tone and context stimuli were

impaired (Bailey et al., 1999). Contextual fear and inhibitory avoidance memory were both impaired by anisomycin infusions into the hippocampus prior to training (Suzuki et al., 2004; Quevedo, 1999). The object recognition memory paradigm takes advantage of a rat's natural tendency to explore novel objects. During training the rats are presented with a number of objects that they will explore. During testing a new object is presented along with the previously explored object. The time spent exploring the new object compared to the previously encountered object is used as a measure of the rats' memory. Object recognition memory was also found to require protein synthesis in the hippocampus; anisomycin infused immediately or three hours after training impaired subsequent recall (Rossato et al., 2007). It could be argued that the above studies result from a retrieval deficit (i.e. a memory was formed but is not able to be retrieved), rather than protein synthesis inhibitors blocking the consolidation process. However, this was shown not to be the case. Hardt et al. (2009) demonstrated that protein blockade in the hippocampus results from a storage deficit rather than a retrieval deficit. They took advantage of the fact that NMDAR are needed for the first but not a second learning trial of contextual fear. The NMDAR inhibitor APV disrupted a second learning trial when anisomycin had been administered immediately after the first learning trial, indicating that anisomycin had impaired consolidation of the first context memory trial and not its subsequent retrieval (Hardt et al., 2009).

1.3.2.2 Consolidation and Molecular Pathways

As highlighted above, LTM formation is dependent on *de novo* protein synthesis. Extensive work has been undertaken to understand the molecular pathways that lead to this synthesis of new proteins during memory consolidation. The first molecular pathways and molecules to be linked to memory formation were again discovered for simple learning in *Aplysia*. Long term facilitation sets in action the cyclic adenosine monophosphate/protein kinase A (cAMP-PKA) molecular pathway (see Fig 1.4). The second messenger cAMP was found to be required for facilitation (Schacher et al., 1988; Scholz

and Byrne, 1988). cAMP activates PKA which moves to the nucleus where it activates the transcription factor cAMP response element binding protein (CREB). CREB binds to cAMP response element (CRE) in the DNA promoter region, leading to protein synthesis (Dash et al., 1990; Bartsch et al., 1998). CREB can also be activated by the phospholipase C (PLC) pathway (see Fig 1.4). In this pathway, PLC hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) to inositol trisphosphate 3 (IP₃) and diacylglycerol (Dg) which act as second messengers (Berridge, 1984). Dg activates protein kinase C (PKC) whereas IP₃ is liberated and binds to its receptors located in the endoplasmic reticulum, leading to intracellular release of Ca²⁺ and CREB activation.

These molecular pathways have also been found to be important in fear conditioning in mice and rats. Intraventricular infusion of inhibitors of PKA activity impaired contextual and auditory fear memory (Schafe et al., 1999). To explore the role of PKA in the hippocampus during auditory fear conditioning, transgenic mice, in which PKA is knocked down in the hippocampus, were developed. It was found that L-LTP was reduced in the CA1 of these mice and contextual and cued fear LTM, but not STM, was impaired (Abel et al., 1997). Infusions of H89, a PKA inhibitor, into the hippocampus immediately after fear conditioning impaired memory twenty four and forty eight hours later, whereas infusions of bucladesine, a cell permeable analog of cAMP, improved memory (Nassireslami et al., 2013). In another study, injecting 8-Bromoadenosine-3',5'-cyclic monophosphate (8Br-CAMP; a degradation resistant activator of PKA) into the hippocampus immediately after IA training using a weak footshock increased LTM two, seven and fourteen days later (Rossato et al., 2009). Injecting PK1 (an inhibitor of PKA) after training with a strong footshock on the other hand, decreased LTM at these time points. Interestingly, if 8Br-CAMP or PK1 are given twelve hours rather than immediately after training then fear memory is only affected at the seven and fourteen day time points (Rossato et al., 2009). 8Br-CAMP infusions into the hippocampus three or six hours after inhibitory avoidance training also improved memory, whereas KT5720 (a PKA inhibitor)

impaired it (Bernabeu et al., 1997). The amygdala also uses the cAMP-PKA pathway as infusions of Rp-cAMPS (an inhibitor of PKA activity) into the laAMY following auditory fear conditioning disrupted freezing levels twenty four hours later (Schafe and LeDoux, 2000). The BLA but not the cAMY nucleus of the amygdala has also been shown to require PKA and PKC during contextual fear memory formation (Goosens et al., 2000). CREB is activated by phosphorylation on Ser 133. Increased levels of phosphorylated CREB (pCREB) compared with controls were found three to six hours after contextual fear learning in the hippocampus and amygdala of mice (Stanciu et al., 2001). Levels of pCREB were elevated immediately, three and six hours after training in the hippocampus (Bernabeu et al., 1997). In a recent study blocking NMDARs in the hippocampus prior to CFC led to decreased levels of pCREB in the amygdala (de Oliveira Coelho et al., 2013). Contextual and auditory fear consolidation was impaired in transgenic mice in which CREB repression was reversibly induced in specific regions including the hippocampus and amygdala (Kida et al., 2002). Transgenic mice in which CREB had been knocked out were found to have reduced L-LTP in the hippocampus and impaired long term contextual and cued auditory fear memory (Bourtchuladze et al., 1994). Infusion of lentivirus vectors containing a mutant form of CREB into the dHC also resulted in impaired contextual fear in rats (Kathirvelu et al., 2013). However, contextual fear is not always impaired by disruption of hippocampal CREB, indicating that CREB-independent fear memory formation is possible, perhaps due to compensation by other transcription factors or proteins (Balschun et al., 2003; Pittenger et al., 2002).

1.3.2.3 Consolidation and Structural Change

Changes to synaptic structure have been found to occur during consolidation. For example, increased varicosities and arbors were found in sensory neurons in *Aplysia* following fear learning (Bailey and Chen, 1988) and changes to dendrite spines occur in the laAMY following fear conditioning (Ostroff et al., 2010). Local protein synthesis has been shown to take place resulting in

structural changes that are specific to activated, rather than non-activated, *Aplysia* synapses (Martin et al., 1997). This local protein translation has been visualized directly using fluorescent translational reporters (Wang et al., 2009). The synaptic tagging and capture hypothesis postulates that during the induction of E-LTP active synapses are 'tagged' which allow them to later receive plasticity related proteins (PRP), enabling E-LTP to be converted to L-LTP (Frey and Morris, 1997; Martin et al., 1997). Evidence for this was first demonstrated in rat hippocampal slices and in cultured *Aplysia* neurons. Using hippocampal slices, electrodes were positioned to stimulate three different synapses designated S1, S2 and S3; both S1 and S2 were from the same neuronal population, whereas S3 was from a separate neuronal population. Firstly S1 was stimulated to induce L-LTP. S2 and S3 were then stimulated but this time in the presence of the protein synthesis inhibitor anisomycin. It was found that L-LTP occurred in S2 but not in S3. This indicated that S2 had been tagged and that protein synthesis produced for L-LTP at S1 could be captured for L-LTP induction of S2 from the same neuronal population, but not S3 from a different neuronal population. Synaptic capture also occurs in *Aplysia* neurons. Pulses of serotonin to one synapse can lead to long term facilitation at another synapse which has received only one pulse of serotonin and would normally only lead to short term facilitation (Martin et al., 1997). Serotonin pulses applied to the soma, which do not normally lead to long term facilitation, can result in long term facilitation at a specific synapse if that synapse also receives one pulse of serotonin (Casadio et al., 1999).

1.3.3 The Neurobiology of Retrieval

Studies investigating the involvement of NMDA and AMPA receptors along with the molecular pathways thought to be important in memory retrieval have been outlined. The retrieval deficit has also been discussed.

1.3.3.1 Retrieval and the Involvement of NMDA and AMPA Receptors

There have been mixed reports looking at the involvement of NMDA and AMPA receptors in memory retrieval. Some studies have found that AMPARs are involved in the retrieval of fear in the BLA and that NMDARs are not. Infusion of the AMPAR inhibitor 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) into the BLA prior to auditory fear memory reactivation was found to block retrieval whereas infusions of the GluN2B antagonist ifenprodil and the NMDAR antagonist APV prior to reactivation did not (Ben Mamou et al., 2006). Intra-hippocampal infusion of CNQX impaired IA retrieval whereas APV infusion had no effect (Szapiro et al., 2000). Milton et al. (2013) also found that AMPARs (using the AMPAR antagonist LY293558) in the BLA were important for the retrieval of auditory fear memory. The AMPAR antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX) was also found to block the retrieval of auditory fear when infused into the BLA or cAMY. The NMDAR antagonist APV on the other hand, did not block retrieval (Zimmerman and Maren, 2010). Intra-dHC infusion of APV did not affect the retrieval of contextual fear (Quinn et al., 2005). Retrieval of taste aversion memory, in which a novel food is associated with stomach malaise (induced by injection of LiCl), was also found to be dependent on AMPARs in the BLA (Rodriguez-Ortiz et al., 2012; Garcia-DeLaTorre et al., 2014). Episodic-like memory retrieval was also found to be dependent on AMPARs but not NMDARs in the hippocampus (Day et al., 2003; Bast et al., 2005). Likewise, object recognition memory retrieval depended on AMPARs but not NMDARs in the perirhinal cortex (Winters and Bussey, 2005). However, in experiments using the context signal model in the *Chasmagnathus* crab, in which retrieval and expression can be dissociated, the opposite was found. AMPARs were not found to be involved in expression whereas NMDARs were. This was

shown by the fact that systemic CNQX did not impair expression, whereas APV did (Barreiro et al., 2013). The context signal model involves placing the crabs individually into the training context, for example a round container. A visual danger stimulus is then moved above the animal in both directions, this is repeated a number of times during which the crabs' behaviour switches from an initial escape response to freezing behaviour. The reduction in the crabs' movement is used as a measure of fear (Barreiro et al., 2013). Other studies have also found using APV that NMDA receptors in the BLA are important for the retrieval of contextual fear (Maren et al., 1996b), auditory fear (Lee et al., 2001) and FPS (Fendt, 2001). However APV impairs both GluN2A and GluN2B containing NMDARs. Another study found that GluN2B expressing NMDAR were not important in the retrieval of auditory or context fear in the BLA (Rodrigues et al., 2001). Therefore it would seem that, in the BLA at least, AMPARs are important for the retrieval of fear whereas NMDARs containing the GluN2B subunit are not. In other studies systemic application of non-specific NMDAR antagonists (e.g. MK-801 and APV) have been found to impair memory, indicating that NMDAR could be important for memory retrieval in brain structures other than the BLA.

1.3.3.2 Retrieval and Molecular Pathways

CREB and PKA have been implicated in the retrieval process. Increases in activated pCREB were found in the amygdala following cued fear retrieval (Hall et al., 2001). In another study neurons in the laAMY were found to be recruited into a memory trace if they had high CREB activity at the time of auditory fear conditioning (Han et al., 2007). Fear retrieval was found to be impaired if these neurons were specifically ablated (Han et al., 2009) and activating these neurons was found to be sufficient to recall a fear memory without behavioural cues (Kim et al., 2014). Increases in pCREB were not found in the hippocampus following cued fear (Hall et al., 2001) or inhibitory avoidance retrieval (Szapiro et al., 2000). However it was found that infusions of the PKA inhibitor Rp-cAMP into the hippocampus impaired retrieval of

inhibitory avoidance whereas PKA activator SP-cAMP enhanced it (Szapiro et al., 2000).

1.3.3.3 The Retrieval Deficit

Many studies struggle to definitively show that agents that induce amnesia prior to or immediately after initial learning or reactivation sessions are affecting the actual memory trace, rather than just resulting in an inability to retrieve an otherwise intact memory; this is known as a retrieval deficit. Recent studies have started to investigate this and have found that memory retrieval can be dissociated from consolidation, reconsolidation and destabilization. For example, it has been demonstrated that protein synthesis blockade of CFC using anisomycin in the dHC results in a storage deficit rather than a retrieval deficit (Hardt et al., 2009). Infusions of the GABA_A receptor agonist muscimol into the perirhinal cortex blocked retrieval of object recognition memory. However, reconsolidation of object recognition was impaired with anisomycin infusion even when retrieval was impaired with muscimol, indicating that retrieval and reconsolidation are independent processes (Balderas et al., 2013). AMPARs were found to be important for the retrieval of auditory fear but not for its destabilization (Ben Mamou et al., 2006; Milton et al., 2013).

1.3.4 The Neurobiology of Reconsolidation

New protein synthesis, NMDARs and activation of macromolecules such as CREB have been proposed to be important in the reconsolidation process, which is similar to findings for consolidation. These have been outlined below along with studies looking at the potential functional role of reconsolidation.

1.3.4.1 Reconsolidation Requires Protein Synthesis

LeDoux and colleagues were the first to show that the process of reconsolidation requires *de novo* protein synthesis (Nader et al., 2000). They found that reactivating a conditioned fear memory and then infusing anisomycin into the BLA resulted in amnesia. After initial fear conditioning,

they reactivated the fear memory by presenting the CS alone (a tone) the following day and then injected anisomycin. The freezing behaviour of the rats was tested twenty four hours later and found to be reduced compared with vehicle injected controls (Nader et al., 2000). They then investigated whether the time at which the anisomycin was infused after reactivation was important and found that if it is given six hours after recall, as opposed to immediately after, no effect was observed indicating that like consolidation, reconsolidation requires time-dependent protein synthesis (Nader et al., 2000). This work initiated a renewed interest in the process of reconsolidation and led to the publication of numerous other studies. Reconsolidation of contextual fear memory was blocked following systemic (Suzuki et al., 2004), intra-amygdala (Parsons et al., 2006), and intra-hippocampal (Debiec et al., 2002; Lee et al., 2004) administration of anisomycin. Reconsolidation of IA memory however, was found to be dependent on protein synthesis in the BLA (Milekic et al., 2007) but not the hippocampus (Taubenfeld et al., 2001). Reconsolidation in *Aplysia* was also found to require protein synthesis as injection of emetine immediately following reactivation impaired the siphon withdrawal reflex (Lee et al., 2012). However, systemic anisomycin was not found to impair reconsolidation of Pavlovian conditioned approach (PCA) using a sucrose reward (Blaiss and Janak, 2007). In PCA, presentation of 10% sucrose solution is paired with a neutral stimulus (CS⁺; i.e. a change of cage lighting). Memory was tested by presentation of the CS⁺ or a CS⁻ (i.e. a tone not associated with sucrose reward) and measuring entries into the sucrose delivery port (Blaiss and Janak, 2007).

1.3.4.2 Reconsolidation and NMDA Receptors

NMDAR have been reported to be important in memory reconsolidation. A number of studies have shown that systemic, intra-amygdala and intra-hippocampal administration of NMDAR antagonists prior to reactivation impaired reconsolidation. In one study, appetitive goal-tracking was used to investigate the effect of different training levels on reconsolidation (Reichelt

and Lee, 2012). In this paradigm two auditory stimuli (i.e. a click and a tone) were used to designate the CS⁺ and CS⁻, where the CS⁺ was always paired with delivery of three sucrose pellets into a magazine port located in the chamber, whereas the CS⁻ was never reinforced. Reactivation sessions consisted of three unrewarded presentations of the CS⁺ and CS⁻. PR-LTM was measured twenty four hours later by recording the number of entries into the magazine during the CS⁺ presentation. Systemic administration of the NMDAR antagonist MK-801, prior to reactivation sessions, impaired PR-LTM if six, but not twelve or three, days of training were undertaken (Reichelt and Lee, 2012). Systemic MK-801 prior to appetitive memory reactivation was also found to impair reconsolidation in another study (Lee and Everitt, 2008). Systemic MK-801 prior to cued auditory fear reactivation was found to disrupt PR-LTM, whereas systemic and intra-amygdala administration of the NMDAR partial agonist D-cycloserine enhanced it (Lee et al., 2006). Systemic administration of MK-801 and intra-amygdala administration of another NMDAR antagonist, D-APV, impaired drug-seeking associative memory when given before reactivation (Milton et al., 2008). Systemic administration of the NMDAR antagonist D-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid prior to reactivation impaired contextual fear memory reconsolidation in mice (Suzuki et al., 2004) and D-APV infused into the dHC prior to reactivation was found to impair contextual fear memory in rats (Lee and Hynds, 2013).

There have been mixed findings when NMDAR antagonists were administered immediately after reactivation, rather than before. Systemic MK-801 immediately after reactivation impaired reconsolidation of contextual fear in mice (Charlier and Tirelli, 2011) and appetitive goal tracking in rats (Reichelt et al., 2013). However, in another study systemic MK-801 immediately after appetitive memory reactivation did not impair reconsolidation (Lee and Everitt, 2008) and infusion of D-APV immediately after reactivation into the amygdala did not impair drug-seeking associative memory (Milton et al., 2008). MK-801 has been shown to exhibit drug-induced state dependency effects. Flint et al., (2013) found that MK-801 given immediately after

reactivation of passive avoidance impaired retention twenty four hours later when tested drug free. However, when MK-801 was given after reactivation and then again twenty minutes prior to a twenty four hour retention test, memory was this time unaffected, indicating state dependency. Caution should therefore be taken when interpreting studies using this drug (Flint et al., 2013).

1.3.4.3 Reconsolidation and Molecular Pathways

There is evidence to suggest the involvement of both PKA and CREB in the reconsolidation process. PKA activation in the BLA enhanced reconsolidation of auditory fear memory, whereas reconsolidation was impaired by PKA inhibition (Tronson et al., 2006). Infusion of Rp-CAMP (PKA inhibitor) into the BLA of rats before reactivation reduced reconsolidation of conditioned taste aversion, leading to quicker onset of extinction (Koh and Bernstein, 2003). Intra-amygdala blockade of PKA immediately, but not six hours after, reactivation also impaired reconsolidation of cocaine associative memory (Sanchez et al., 2010). PKA activity was found to be increased in the snail *Lymnaea* if memory was reactivated six hours, but not twenty four hours, after associative conditioning. Moreover, PKA inhibition six hours but not twenty four hours after training impaired reconsolidation (Kemenes et al., 2006). Contextual and auditory fear reconsolidation was impaired in transgenic mice in which CREB repression could be reversibly induced in specific regions, including the hippocampus and amygdala (Kida et al., 2002). Contextual fear memory reconsolidation was found to be impaired in transgenic mice in which CREB function was disrupted. pCREB was increased in the hippocampus and amygdala but not the mPFC following reactivation (Mamiya et al., 2009).

1.3.4.4 Role for Reconsolidation

Although reconsolidation has been shown to occur in many instances, not all memories undergo the process. Such situations are referred to as boundary conditions and include the strength and age of the memory (Lee, 2009). For

example, it has been shown that weaker memories are more likely to undergo reconsolidation than strong memories (Suzuki et al., 2004; Rodriguez-Otíz et al., 2008). It has also been shown for some memory paradigms (e.g. inhibitory avoidance) that older memories are less likely to undergo reconsolidation than weaker ones (Milekic and Alberini et al., 2002). Boundary conditions could be argued to support a proposed role for reconsolidation in memory updating, thereby enabling the relevance of a memory to be maintained (Lee, 2009). Lee and colleagues provided further evidence supporting this idea. By taking advantage of the doubly dissociable molecular mechanisms underlying consolidation (BDNF) and reconsolidation (Zif268), they found that fear memory strengthening that occurred as a result of a second learning trial was dependent on reconsolidation rather than consolidation mechanisms. Knockdown with Zif268 antisense oligodeoxynucleotides blocked the increase in memory strength, whereas BDNF antisense oligodeoxynucleotide infusion did not (Lee, 2008). It has also been shown that reconsolidation is required to update a neutral contextual memory to a contextual fear memory in the dHc (Lee, 2010). This experiment made use of the context pre-exposure facilitation effect. In this paradigm rats are placed into a novel chamber in order to gain familiarity to it. The next day the rats are returned to the chamber (which reactivates the contextual memory) and immediately given a footshock. An association between the reactivated contextual memory and the footshock is learnt so that in a subsequent re-exposure test the rat exhibits increased freezing behaviour (Biedenkapp and Rudy, 2004). By infusing either BDNF or Zif268 antisense oligodeoxynucleotides it was shown that reconsolidation (Zif268) but not consolidation (BDNF) mechanisms are required for updating hippocampal memory (Lee, 2010).

1.3.5 The Neurobiology of Destabilization

Studies have found that destabilization is dependent on protein degradation and glutamate receptor signalling. It has been proposed that prediction error could determine whether or not memories will be destabilized and therefore undergo reconsolidation. This is discussed in more detail below

1.3.5.1 Destabilization Requires Protein Degradation

Destabilization has been shown to require ubiquitin proteasome-dependent protein degradation. The ubiquitin proteasome system involves the tagging of proteins by ubiquitin and their degradation by proteasomes, which are a complex of enzymes able to break peptide bonds. Injection of clastolactacystin β -lactone (an ubiquitin proteasome inhibitor) into *Aplysia* following memory reactivation prevented PR-LTM impairment produced by the protein synthesis inhibitor emetine (Lee et al., 2012). This was found to take place at the same sensory-motor neuron synapse in which the original learning occurred (Lee et al., 2012). Ubiquitin/proteasome-dependent protein degradation was found to be important for contextual fear memory destabilization in the CA1 region of mice (Lee et al., 2008). Protein degradation was also found to occur during cocaine conditioned place preference (CPP) destabilization in the nucleus accumbens core but not shell (Ren et al., 2013). During CPP the rats learn to associate a certain context with a drug reward, such as amphetamine, morphine or cocaine. They show this by spending more time in the drug, compared to the vehicle, associated area when tested later without drug present.

1.3.5.2 Destabilization and the Involvement of NMDA and AMPA Receptors

The GluN2B subunit of the NMDAR has been shown to be important in memory destabilization. In one study, the GluN2B antagonist ifenprodil was infused into the BLA prior to auditory fear reactivation and was found not to impair reconsolidation, whereas anisomycin infusion immediately after did. However, when ifenprodil was administered before reactivation and anisomycin immediately after, PR-LTM was unaffected. This indicated that blocking the GluN2B subunit before reactivation prevented destabilization as the memory trace was not vulnerable to manipulation by anisomycin (Ben Mamou et al., 2006). Another study also found that the GluN2B subunit was important for auditory fear memory destabilization in the BLA (Milton et al., 2013). A double dissociation between GluN2B and GluN2A was established, with GluN2B shown to be required for destabilization and the GluN2A subunit

for memory reconsolidation (Milton et al., 2013). AMPAR have been found to participate in memory destabilization, with the transient exchange from Ca²⁺ impermeable AMPAR (GluA2 containing) to Ca²⁺ permeable AMPAR (no GluA2) having been shown to underlie destabilization of auditory fear memory in the laAMY (Hong et al., 2013).

1.3.5.3 Destabilization and Prediction Error

Prediction error occurs when there is discrepancy between expected and actual events. Prediction error can be either positive or negative. If a rat has previously undergone CFC and is returned to the conditioning chamber it would expect to receive a footshock in that context; if it does not, then arguably a negative prediction error could have occurred. In regard to studies investigating reward, prediction error is positive if a reward is surprising or unexpected, whereas a negative prediction error occurs where a predicted reward is absent (Schultz, 2000). Prediction errors can also be influenced by probability (e.g. what is the likelihood of gaining a reward) and by temporal elements (e.g. does the reward occur when expected or is it delayed) (Schultz, 2000). The presence of a prediction error would indicate that the memory needs to be updated, as the previously learnt association is no longer relevant. Prediction error could potentially be important for modifying an existing memory trace, which occurs through the process of reconsolidation. In order for a memory to undergo reconsolidation the original trace needs to be destabilized. It is possible that the occurrence of a prediction error determines whether or not a memory will be destabilized (Sevenster et al., 2013). Prediction error was found to be a requirement for fear memory reconsolidation in human subjects and was proposed to be a potential index for memory destabilization. Reconsolidation of FPS was impaired by administration of propranolol, a beta-adrenergic antagonist that disrupts reconsolidation in several different paradigms, after reactivation but only in subjects where either positive or negative prediction error occurred. In this experiment a positive prediction error was created by using partially reinforced training sessions (e.g. participants only received shocks some of

the time during the CS presentation and were then reinforced with a shock during the reactivation session). The negative prediction error was created by always pairing the shock and CS together during training but not during the reactivation session (Sevenster et al., 2013). In another study temporal prediction errors were also found to be important in determining whether or not a memory would be destabilized (Diaz-Mataix et al., 2013). The timing between the CS and onset of the US was increased or decreased in duration compared with the timing used during training, therefore creating a temporal prediction error. It was found that reconsolidation took place under conditions where temporal prediction error occurred, even for strong memories which can be subject to boundary conditions (see above), and that this could be blocked by infusions of anisomycin into the laAMY of rats (Diaz-Mataix et al., 2013).

1.3.6 The Neurobiology of Extinction

Extinction, like consolidation, has been shown to require NMDAR activation, protein synthesis, and macromolecules such as CREB. However more recent work has shown that weakening of the original memory also takes place in the form of reversed LTP or depotentiation (Pape and Pare, 2010). These studies have been outlined below.

1.3.6.1 Extinction and NMDA Receptors

Acquisition of extinction, like acquisition of other types of memory, has been shown to require NMDARs. Systemic administration of the NMDAR antagonists D-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid or MK-801 impaired the extinction of contextual and auditory fear respectively (Suzuki et al., 2004; Baker and Azorlosa, 1996). Systemic or intra-BLA administration of the NMDAR agonist D-cycloserine was found to enhance auditory fear extinction (Lee et al., 2006). APV infused into BLA prior to non reinforced tone presentations blocked extinction of auditory fear, but not when infused into the cAMY (Zimmerman and Maren, 2010). FPS extinction

also seems to be dependent on NMDARs in the BLA. APV infusions were found to impair extinction (Falls et al., 1992) whereas D-cycloserine was found to enhance it (Walker et al., 2002). Intra-BLA or dHC infusion of APV impaired extinction of inhibitory avoidance (Myskiw et al., 2010). Systemic administration of the GluN2A antagonist NVP-AAM077 did not impair extinction of auditory fear, whereas the GluN2B antagonist Ro25-6981 did (Dalton et al., 2012). It would seem that the laAMY and that GluN2B-containing NMDARs (as opposed to GluN2A-containing NMDARs), are important for the acquisition of auditory fear extinction. Ifenprodil (a GluN2B specific antagonist) infused into laAMY, but not the mPFC, prior to the extinction session impaired subsequent retention of auditory fear extinction (Sotres-Bayon et al., 2007). Intra-laAMY infusions of ifenprodil immediately after extinction training did not impair extinction whereas intra-mPFC infusions did (Sotres-Bayon et al., 2009). This indicates that GluN2B-containing NMDARs within the laAMY are important for the acquisition but not the consolidation of auditory fear extinction, whereas in the mPFC GluN2B-containing NMDARs are important for consolidation of extinction but not its acquisition (Sotres-Bayon et al., 2009). NMDARs were also found to be needed in the dHC for the extinction of IA (Szapiro et al., 2003).

1.3.6.2 Extinction Requires Protein Synthesis

Extinction consolidation, similar to other types of consolidation, has been shown to depend on protein synthesis in a number of brain regions depending on the type of memory paradigm used. Application of the protein synthesis inhibitor cycloheximide one hour prior to a one hour extinction session reduced context fear consolidation in the *Chasmagnathus* crab (Pedreira and Maldonado, 2003). Consolidation of auditory fear extinction was also found to require protein synthesis. Infusions of anisomycin prior to extinction training into the lateral ventricle or the prefrontal cortex (PFC), but not the insular cortex of rats, blocked extinction retention the following day (Santini et al., 2004). It would appear that the extinction of conditioned taste aversion however, does require protein synthesis in the insular cortex, as rats infused

with anisomycin prior to extinction sessions were found to have reduced extinction memory (Berman and Dudai 2001). Protein synthesis also seems to be required in the BLA and hippocampus for fear memory extinction. Anisomycin infused into the BLA of rats prior to light alone presentations decreased FPS extinction (Lin et al., 2003b). Rats infused with anisomycin into the hippocampus immediately after the first of three retention tests showed impaired IA extinction compared with saline injected controls (Vianna et al., 2001). In another study intra-hippocampal protein synthesis inhibition was found to accelerate extinction (Fischer et al., 2004). However, it could be argued that the drug infusions administered after the first three minute extinction session used in this study would have resulted in reconsolidation mechanisms being targeted rather than extinction.

1.3.6.3 Extinction and Molecular Pathways

CREB and PKA have been implicated in fear memory extinction. Inhibitory avoidance extinction was impaired by intra-dHC infusions of Rp-cAMP (PKA inhibitor) before or after extinction training (Szapiro et al., 2003). Intra-BLA or dHC infusion of Rp-cAMP also impaired acquisition of inhibitory avoidance extinction. Increases in phosphorylated PKA were found in the dHC, but not the BLA, following extinction training (Myskiw et al., 2010). Increases in pCREB were found in the mPFC and amygdala, but not the hippocampus, following contextual fear extinction training and reduction of CREB mediated transcription using CREB repressor mice impaired extinction (Mamiya et al., 2009).

1.3.6.4 Extinction and Synaptic Depotentiation

Although it is generally accepted that extinction involves the formation of a new memory that competes with the original one, there is some evidence to suggest that extinction also results in some weakening of the original memory trace. Depotentiation, a reversal in LTP, has been linked to memory extinction along with kinase dephosphorylation, impaired AMPAR endocytosis and

changes in dendrite structure. Studies investigating these have been outlined below.

Low frequency stimulation was found to result in depotentiation at the EC-lamyg synapse in rat brain slices. Application of cyclosporine A (inhibitor of calcineurin) was found to impair depotentiation, most likely by inactivating protein kinases important for synaptic plasticity. *In vivo* quenching stimulation (low frequency lamyg stimulation) reduced FPS extinction in rats and systemic cyclosporine A administration prior to quenching stimulation impaired this (Lin et al., 2003a). Calcineurin was increased in rat amygdala following FPS extinction and inhibitors of calcineurin, including cyclosporine A and FK-506, impaired extinction (Lin et al., 2003b). Systemic administration of Tat-GluR23Y (a peptide that blocks the endocytosis of AMPAR) prior to unreinforced CS presentations was found to impair the acquisition of auditory fear extinction (Dalton et al., 2008). Systemic or intra-amygdala administration of 4-[2-(phenylsulfonylamino) ethylthio]-2,6-difluorophenoxyacetamide (a potentiator of AMPARs) enhanced extinction but not reconsolidation of contextual fear in mice (Yamada et al., 2009). Potentiation at the thalamic synaptic input to the lamyg was found to be reversed in brain slices taken from auditory fear extinction-trained rats. Infusions of the Tat-GluR23y peptide into the lamyg prior to a tone alone session impaired extinction (Kim et al., 2007). Changes in dendritic spines were found to be altered in the frontal association cortex of mice following auditory fear training or extinction using transcranial two-photon microscopy; spines located on the same dendritic branch were either increased or decreased following either extinction or fear conditioning, respectively (Lai et al., 2012). Extinction along with the other learning and memory stages has been linked with DA transmission. DA and its involvement in these processes, has therefore been outlined in the following sections.

1.4 Dopamine Transmission

Previous research investigating DA transmission in memory acquisition, consolidation, retrieval, reactivation, destabilization and extinction has produced mixed results. Some of this research has been outlined, along with studies investigating DA biosynthesis, metabolism and signalling. The research in this thesis focuses on the involvement of D1 receptors (D1R) in the amygdala and hippocampus, studies showing the distribution of D1Rs and DA innervations of these brain regions have therefore also been outlined.

1.4.1 Dopamine Biosynthesis and Metabolism

DA was first identified as a neurotransmitter, rather than just a precursor for noradrenaline (NA), by Arvid Carlsson and colleagues (Carlsson et al., 1962). DA is a monoamine and member of the catecholamine family. It is synthesised by the enzymes tyrosine hydroxylase (TH) and DOPA decarboxylase from tyrosine. Tyrosine hydroxylase converts tyrosine to L-DOPA, which, in turn is converted by DOPA decarboxylase into DA. Once synthesized, DA is packaged into vesicles and released under conditions of depolarization. DA is transported from the extracellular space in the synapse to the pre-synaptic terminal by the dopamine transporter (DAT), thereby controlling the levels of available DA in the brain. The DAT is a plasma membrane protein with twelve transmembrane spanning helices, helices one and six form the active site. The DAT is a member of the solute carrier six transporter family of neurotransmitter transporters. The DAT couples inward DA transport with the movement of Na^+ and Cl^- down their electrochemical gradient (Vaughan and Foster, 2013; Pramod et al., 2013; Schmitt and Reith, 2010). Once back in the neuron DA is either repackaged into vesicles for reuse or metabolised into dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) by monoamine oxidase B (MAO) or catechol-O-methyl transferase (COMT), respectively.

1.4.2 Dopamine Innervations of the Amygdala and Hippocampus

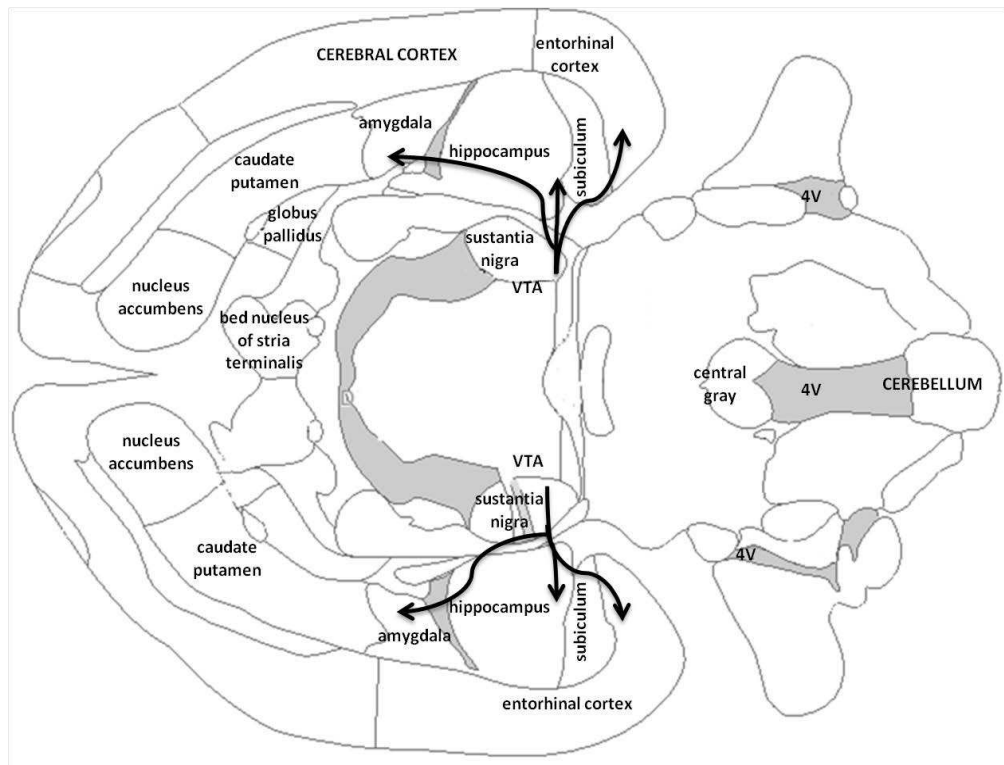


Fig 1.3 Horizontal section of rat brain showing DA innervations of the amygdala complex and hippocampal formation (modified from Paxinos and Watson, 2009)

Dahlstroem and Fuxe (1964) were the first to determine the main catecholamine projections, DA and NA, in the brain and named them A1-A12. They used the Falck-Hillarp technique, which utilizes formaldehyde vapour to convert DA and NA to isoquinoline molecules which can then be visualized as a yellow-green fluorescence. Dopaminergic neurons located within the midbrain were found in the retrorubral area, the substantia nigra (SN) and ventral tegmental area (VTA) and were designated A8, A9 and A10, respectively. Early studies found that the neurons originating in the SN and retrorubral area formed the nigrostriatal pathway projecting to striatal regions such as the putamen and caudate nucleus, whereas neurons originating within the VTA formed the mesolimbic and mesocortical pathways

projecting to structures including the nucleus accumbens, amygdala, hippocampus and PFC. However, the advance of more refined staining techniques such as immunohistochemistry, which can stain enzymes important in the synthesis of dopamine such as TH, has shown this to be an oversimplification (Bjorklund and Dunnett, 2007). The SN not only contributes projections to the nigrostriatal pathway but also to the mesolimbic and mesocortical pathways and the VTA projects to the striatum as well as limbic and cortical structures (Fallon and Moore, 1978; Swanson, 1982). Studies using TH immunohistochemistry have revealed that the lateral division of the cAMY, the BLA and the IPCs receive substantial dopaminergic innervations from the SN-VTA complex (Asan, 1997; Brinley-Reed and McDonald, 1999; Fuxe et al., 2003; Pinard et al., 2008). The IPCs and lateral division of the cAMY contain inhibitory interneurons which are important for the trafficking of information from the BLA to the medial cAMY and receive the highest DA innervations. DA is therefore ideally placed to modulate this information flow (de la Mora et al., 2010). Retrograde tracing using the fluorescent tracer fluoro-gold and anterograde tracing combined with immunocytochemistry staining for TH showed a modest dopaminergic projection from the SN-VTA complex to the hippocampal formation, with the CA1 and subiculum nuclei receiving the highest innervations (Gasbarri et al., 1994). Initial studies investigating DA projections in humans used Falck-Hillarp fluorescence histochemistry and TH immunohistochemical staining of post-mortem brains and confirmed the A1-A12 distribution of catecholamines (including DA) previously outlined in animals (Olson et al., 1973; Pearson et al., 1979; Pearson et al., 1983). However more recent studies have indicated that the mesolimbic pathway originates from both the SN and VTA and that an anatomical distinction between these regions is less apparent in humans compared with rats (Düzel et al., 2009). Multi-tensor diffusion tensor imaging was used to compare neural connectivity between the SN or VTA and different brain regions in humans. Higher connectivity was found between the SN than the VTA for all brain regions assessed, including the amygdala (Kwon and Jang, 2014).

1.4.3 Dopamine Receptors

DA receptors are comprised of seven transmembrane regions which are linked by protein loops and come together to form the ligand binding site. They also have an intracellular carboxyl tail and are coupled to a GTP-binding protein (G-protein). The initial classification of two families of DA receptors was based on findings from biochemical and pharmacological studies. It was found that while activation of some DA receptors led to adenylate cyclase dependent increases in the synthesis of cAMP, others did not. It was also shown that selective drugs had high affinities for some DA receptors but not others (Kebabian et al., 1972; Kebabian and Calne, 1979). Two classes of dopamine receptors were therefore named the D1Rs and the D2 receptors (D2Rs). Activation of D1Rs leads to increased cAMP levels whereas D2R activation inhibits cAMP synthesis. D1Rs are antagonised by benzazepine ligands such as *R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride* (SCH 23390), whereas D2Rs are antagonised by benzamides such as sulpiride (Kebabian and Calne, 1979). With the advance of molecular techniques in the late 1980s, gene cloning and the elucidation of the protein structures of the dopamine receptors was undertaken. Five different DA receptors were identified and designated D₁-D₅. In this thesis the term D1R is used to describe the D1 receptor family which includes the D₁ and D₅ receptors and the term D2R is used to describe the D2 receptor family which includes the D₂, D₃ and D₄ receptors. D1Rs were found to have no introns (the parts of the gene removed by splicing) in their genes. In contrast, the D2Rs do have introns (Zhou et al., 1990; Sunahara et al., 1991; Tiberi et al., 1991). The protein structures also differ between the two families. For example, the third intracellular loop and the carboxyl tail were found to be longer in the D1R family, compared with the D2R family (Strange, 2000). The classification of DA receptors into D1 and D2-like families, however, is over-simplistic. Recent work has shown that subunits from D1Rs can come together with subunits from the same or different receptor families to form heteromers. For example, the D1R has been shown to form heteromers with the NMDAR. The GluN1 subunit of NMDA receptors can

form direct interactions with the D1R via protein-protein bonds (Nai et al., 2010). These new combinations of receptor subunits can lead to alterations in activation of signalling pathways, can affect ligand binding leading to changes in the potency of agonists, and new binding sites can be created (Missale et al., 2010). D1 and D2 receptors are widely distributed in the brain. They are located in the caudate, putamen, nucleus accumbens olfactory tubercle, SN, VTA, PFC, amygdala complex and hippocampal formation, with the density of D1Rs tending to be greater in most brain regions compared to D2Rs (Neve and Neve, 1997).

1.4.4 Distribution of D1 Receptors in Amygdala and Hippocampus

The localization of D1Rs within the amygdala and hippocampus are outlined in more detail below. Quantitative autoradiographic methods using radioligands such as ³H-SCH 23390 and ³H-SKF-83566 (D1R antagonists) have been used to determine the levels of D1Rs in the rat brain. High densities of D1R were found in the amygdaloid complex, with the ventral BL nucleus and the IPCs having the highest level. D1Rs were also located in the hippocampal complex, including the EC, parasubiculum, hippocampus proper and DG (Boyson et al., 1986; Dawson et al., 1986; Köhler et al., 1991; Scibilia et al., 1992). These studies were later confirmed by immunohistochemical localization of D1R in rat brain using a polyclonal antibody directed to the D1R (Huang et al., 1992; Perez de la mora et al., 2006). *In situ* hybridization histochemistry (using a labelled complementary RNA probe for D1R mRNA) also showed D1R mRNA expression was present in the amygdala and hippocampus (Weiner et al., 1991). Electron microscopy has revealed that the D₁ and D₅ subtypes are both present in the same BLA neurons, with the D₁ subtype predominantly found on the dendritic spines and the D₅ subtype at dendritic terminals (Muly et al., 2009). The rat D1R gene is 92% identical to the human D1R gene (Zhou et al., 1990) and D1Rs are located in the human brain, including in the amygdala and hippocampus (Mengod et al., 1991). Reverse transcription polymerase chain reaction was used to ascertain the levels of D1R mRNA in the different subregions of the human amygdala; in contrast to findings in the rat, the

cAMY was found to have the highest level of D1R expression (Xiang et al., 2008).

1.4.5 D1 Receptor Signalling

DA receptors are coupled to G-proteins which are composed of α , β and γ subunits. D1Rs can be linked with different α subunits including $G\alpha_s$, $G\alpha_{olf}$ or $G\alpha_q$ whereas D2Rs are linked to $G\alpha_i$ or $G\alpha_o$. G-proteins containing the $G\alpha_s$, $G\alpha_{olf}$ or $G\alpha_q$ subunit have been shown to initiate the cAMP pathway (see Fig 1.4). The enzyme adenylate cyclase is activated by D1R stimulation and converts ATP to cAMP, which, in turn, activates PKA, leading to gene expression via transcription factors such as CREB and phosphorylation of DA and cAMP regulated phosphoprotein, 32kDA (DARPP-32). DARPP-32 is then able to inhibit protein phosphatase 1. PKA activation can lead to modulation of ion channels, including K^+ , Ca^{2+} and Na^+ channels (Neve et al., 2004; Undieh, 2010). D2R activation on the other hand results in reduced levels of adenylate cyclase, leading to decreased PKA and DARPP-32 activation (Neve et al., 2004). G proteins containing $G\alpha_q$ can also lead to the activation of the PLC pathway which also results in activation of CREB.

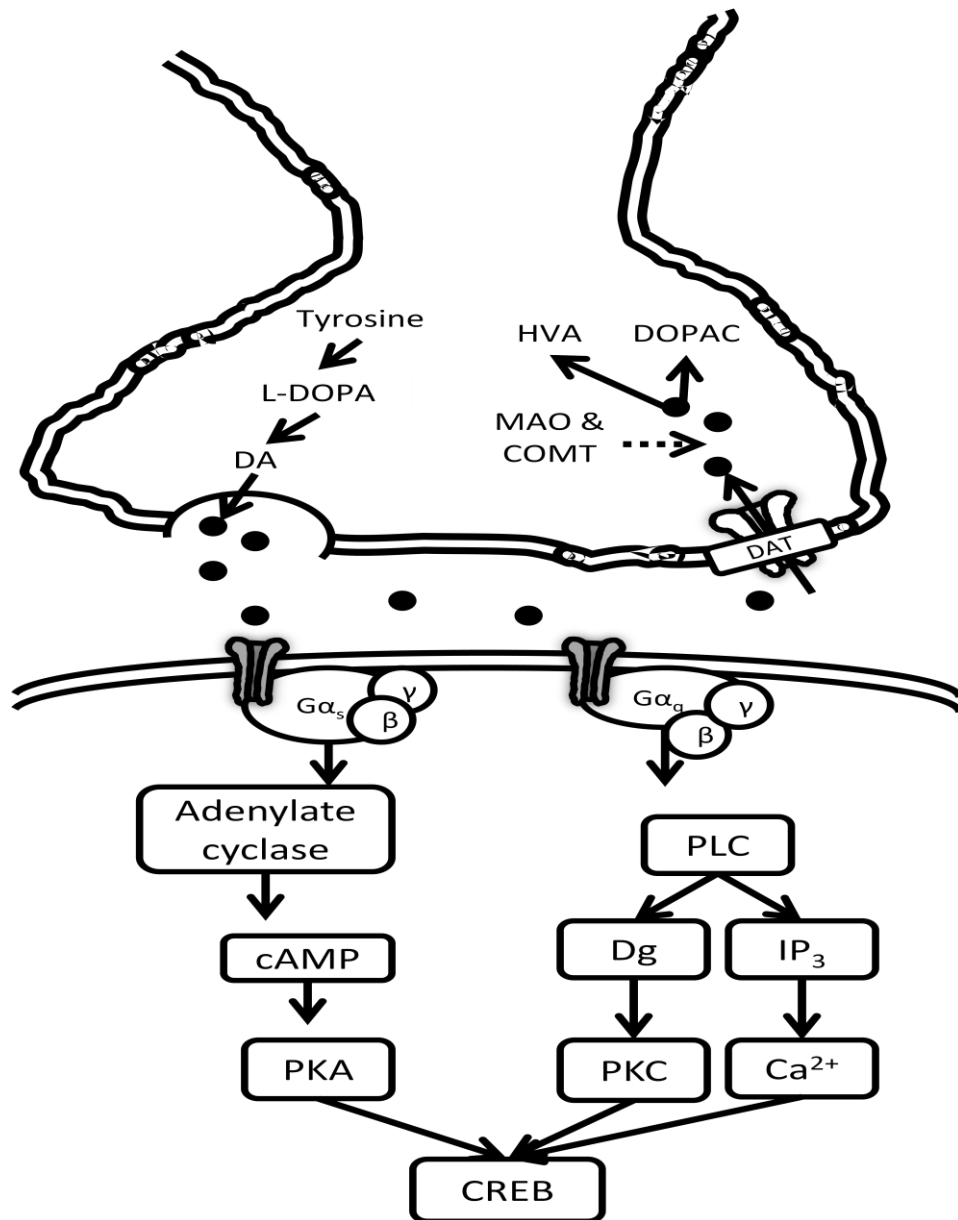


Fig 1.4 D1R signalling

DA is synthesised from tyrosine before being released into the synapse and binding to D1Rs. D1Rs coupled to Gα_s result in activation of the enzyme adenylate cyclase and increased levels of cAMP. PKA is activated which in turn activates CREB. D1Rs coupled to Gα_q stimulated PLC leading to increased Dg and IP₃. Dg activates PKC whereas IP₃ leads to release of Ca²⁺ from the endoplasmic reticulum and activation of CREB. DA is returned to the neuron by the DAT where it is metabolised by COMT or MAO into DOPAC and HVA.

There is evidence to suggest that D1R activation in the amygdala and hippocampus is linked to the PLC pathway and D₅ coupling to G α_q . In the hippocampus and amygdala D1Rs were found to couple to G α_q but not G α_s (Jin et al., 2001). D1R activation was found to increase IP₃ formation in the amygdala and hippocampus (Undie and Friedman 1990). D₁ knockout mice had reduced cAMP production but inositol phosphate accumulation following D1R stimulation was unaffected. Binding of ³H-SCH 23390 to G α_q subunit was also unaffected, whereas G α_s was reduced (Friedman et al., 1997). Conversely, D₅ knockout mice were found to have deficient PLC signalling (Sahu et al., 2009). It was also found that increased intracellular Ca²⁺ was released in cultured hippocampal neurons following D1R activation (Lezcano and Bergson, 2002) and that amygdaloid D1R activation did not increase cAMP (Leonard et al., 2003). SKF 83959 is a selective D1R agonist that does not work by stimulating adenylyl cyclase but instead increases PLC mediated hydrolysis of phosphoinositide (Panchalingam and Undie, 2001). Application of SKF 83959 to cultured hippocampal neurons resulted in a PLC-dependent increase in Ca²⁺ release from internal stores and also increased influx via Ca²⁺ channels (Ming et al., 2006). D1R signalling via G α_q coupling to the PLC pathway has also been demonstrated in the PFC of humans (Pacheco and Jope, 1997).

1.4.6 Dopaminergic Modulation of Memory Acquisition

DA has been shown to be an important modulator of memory acquisition. There is strong evidence from electrophysiological studies to suggest that D1Rs are involved in the induction of LTP *in vitro* and *in vivo*. Application of the D1R agonists, 6-chloro-PB and dihydrexidine, prior to high frequency stimulation increased E-LTP in the CA1 of hippocampal slices whereas SCH 23390 impaired this effect (Otmakhova and Lisman, 1996). L-LTP in the CA1 was also found to be blocked by application of SCH 23390 during but not after, high frequency stimulation (Frey et al., 1991; Huang and Kandel, 1995). In another study, a high frequency stimulation protocol that does not elicit LTP under control conditions was found to result in LTP induction in the

presence of the D1R agonist SKF 38393 in both regular-spiking and burst-spiking subiculum neurons, an effect which was blocked by SCH 23390 application. This LTP induction was also found to be dependent on NMDARs, PKA and post-synaptic Ca^{2+} increases (Roggenhofer et al., 2010; Roggenhofer et al., 2013). Slices taken from the brains of D1R knockout mice showed a reduction in hippocampal LTP (Matthies et al., 1997). D1-NMDA receptor heterodimerization could be important for this as LTP was found to be impaired in cultured hippocampal cells in which D1-GluN1 coupling was disrupted with the interfering peptide D1-t2 (Nai et al., 2010). In a study using awake-behaving rats, *in vivo* recording of LTP at the Schaffer collateral CA1 synapse was undertaken. It was found that intra-ventricular administration of chloro-PB (D1R agonist) assisted the induction of LTP, whereas SCH 23390 blocked this. Short term potentiation (STP) was converted to LTP by exposure of the rats to an empty hole-board apparatus and this was also blocked by SCH 23390 administration (Lemon and Manahan-Vaughan, 2006). Similarly, it was found that LTP could be induced by a weak high frequency stimulation protocol if the rats were allowed to explore a novel environment and this was also blocked by intra-ventricular SCH 23390 administration prior to testing (Li et al., 2003). Reduced LTP was found in the CA3-CA1 Schaffer collaterals following high frequency stimulation in D1R knockout (D1R KO) mice and in control mice injected with small interfering RNA into the hippocampus to knock down D1R expression (Ortiz et al., 2010). LTP has also been found to occur in the amygdala (Rogan et al., 1997). L-LTP can be induced in the laAMY following both low and high frequency stimulation of fibres from the cortex (by stimulating the external capsule) in brain slice preparations (Huang and Kandel, 2007). Application of the D1R antagonist SCH23390 before low frequency stimulation resulted in blockade of L-LTP (Huang and Kandel, 2007). However, in another study LTP induction in the laAMY was found to be modulated by the D2R but not the D1R (Bissiere et al., 2003).

Numerous behavioural studies have investigated the role of DA in different types of memory acquisition including contextual and auditory fear

conditioning, object recognition, spatial, episodic-like memory and appetitive learning (Inoue et al., 2000; Pezze et al., 2002; Clausen et al., 2011; O'Carroll et al., 2006; Bethus et al., 2010; Hiroi and White, 1991). Increased levels of DOPAC were observed in the amygdala following footshock administration (Herman et al., 1982). In another study DA levels in the hippocampus and DOPAC levels in the amygdala were found to be increased after CFC and following placement in the fear conditioning chamber without footshocks twenty four hours later (Inoue et al., 1994). Object recognition was found to be impaired by D1R antagonism (Clausen et al., 2011). Rats injected with SCH 23390 prior to training spent less time exploring a novel object when tested five minutes later compared with saline injected controls (Clausen et al., 2011). D1Rs have also been shown to be important in the delayed matching-to-place (DMP) water maze test. In this test rats are tested on their ability to locate a hidden platform after one learning trial. Swimming path lengths are used as an indication of memory, with shorter path lengths indicating better memory retention. Intra-dHC SCH 23390 fifteen minutes before a new location trial, was found to impair memory in the DMP water maze thirty minutes (Pezze and Bast, 2012) and six hours later (O'Carroll et al., 2006). Episodic-like memory acquisition also requires DA. In one memory paradigm rats were trained to associate a particular flavoured food pellet (e.g. banana) with a particular location in an event arena. The rats then underwent probe tests in which they were either cued with a flavour or were not cued before being placed in the event arena. The time they spent digging at the correct location was used as an indication of memory performance; their episodic-like memory for 'what' and 'where' was therefore being tested. Rats that were infused with SCH 23390 into the dHC prior to learning a new paired association had impaired memory twenty four hours, but not thirty minutes, later (Bethus et al., 2010). It was found that D1R KO mice had impaired spatial learning in the Barnes maze and Morris water maze (Ortiz et al., 2010; EL-Ghundi et al., 1999). In the Barnes maze the mouse is placed on the white circular surface with holes around the periphery, one of which leads to a drop box into which the mouse can escape the exposure of the table top. The

mouse's spatial memory is assessed on its ability to remember the location of the one hole which leads to the drop box. It was also found that these mice had impaired inhibitory avoidance, contextual and auditory fear conditioning (Ortiz et al., 2010) and D1R striatum specific KO mice were found to have impaired CFC (Ikegami et al., 2014). However, in another study, D1R KO mice were found to have normal CFC and inhibitory avoidance acquisition (EL-Ghundi et al., 2001). The DA receptor agonist, apomorphine, was found to enhance two-way avoidance learning (Reis et al., 2004). In this test presentation of a light stimulus is associated with the onset of a footshock. The rats can avoid being footshocked by quickly moving to a safe compartment when the light stimulus is turned on. It was found that apomorphine injected rats learnt faster than the saline-injected controls (Reis et al., 2004). Intra-nucleus accumbens and intra-striatum infusion of SCH 23390 prior to two-way active avoidance training impaired subsequent retention (Wietzikoski et al., 2012). D1Rs have also been implicated in the acquisition of CPP D1R mutant mice were found to have impaired acquisition of cocaine CPP (Chen and Xu, 2010). Systemic administration of SCH 23390 has been shown to block the acquisition of CPP to cocaine (Cervo and Samanin, 1995) and amphetamine (Hiroi and White, 1991). Other studies have investigated the involvement of D1Rs in contextual and auditory fear acquisition. Infusion of the D1R agonist SKF 82958 into the BLA was found to enhance CFC (Biedenkapp and Rudy, 2009). DA antagonists given systemically or centrally have been shown to impair FPS (Greba and Kokkinidis, 2000), auditory fear conditioning (Pezze et al., 2002; Guarraci et al., 1999) and CFC (Inoue et al., 1996; Inoue et al., 2000; Calzavara et al., 2009; Bai et al., 2009). These studies are outlined in greater detail in chapters three and four. It should be noted that due to the substantial DA innervations of the basal ganglia, a brain region important in the production of movement, most DA drugs especially when administered systemically can have non-specific effects on locomotion that could interfere with behavioural performance. For example systemic SCH 23390 has been shown to impair total distance moved

in the open field (Gessa et al., 1985; Salmi and Ahlenius, 2000; Clausen et al., 2011).

1.4.7 Dopaminergic Modulation of Consolidation

There are numerous studies indicating a link between DA and memory consolidation. The protein synthesis-inhibiting drug anisomycin infused into the BLA immediately after auditory fear conditioning impaired memory (Schafe and LeDoux, 2000). It has been argued that anisomycin induced amnesia could be a result of aberrant neurotransmitter release, rather than (or as well as) blockade of *de novo* protein synthesis, as large increases in DA have been shown in both the amygdala and hippocampus following anisomycin infusion (Canal et al., 2007; Qi and Gold, 2009).

Studies using D1R antagonists and agonists have demonstrated the involvement of D1Rs in consolidation in many different memory paradigms. The effects of amphetamine, which causes the release of DA and NA from the pre-synaptic terminal, on the consolidation of morphine CPP was assessed (Blaiss and Janak, 2006). It was found that injecting amphetamine immediately after training resulted in the enhancement of morphine CPP, indicating DA and/or NA involvement in its consolidation (Blaiss and Janak, 2006). CPP using cocaine was also found to result in increased D1R protein and mRNA expression in the hippocampus (Tanaka et al., 2011). In another study the effect of infusing SCH 23390 and SKF 38393 (D1 agonist) into the PFC on the consolidation of object recognition memory was investigated (Maroun and Akirav, 2009). Two groups of rats were used; one group had been habituated to the testing area and so had low arousal, while the other group were non-habituated rats that had high arousal levels and thus increased circulating corticosteroids and higher extracellular levels of DA. They found that the rats that had intermediate levels of D1R activation (i.e. H/SKF38393 and NH/SCH23390 rats) had optimum memory performance (Maroun and Akirav, 2009). It was also found that systemic administration of SKF 38393 immediately after object recognition training enhanced memory,

whereas enhancement by the non-selective DA agonist apomorphine administered immediately after training was blocked when preceded by SCH 23390 treatment, implying D1R involvement (De Lima et al., 2011). Other studies have shown the involvement of D1R in inhibitory avoidance consolidation. It was found that dopamine deficient (DD) and D1R KO mice have impaired short-term (10 min) and long-term (24 hr) inhibitory avoidance memory (Fadok et al., 2009). Restoration of DA levels in DD mice by administration of L-DOPA enabled memory formation if administered immediately after training, indicating the involvement of DA in memory consolidation. DA restoration selectively in the BLA rescued STM, but not LTM, whereas VTA DA restoration rescued both STM and LTM (Fadok et al., 2009).

Methylphenidate (prevents reuptake of DA and NA) administration into the BLA immediately but not six hours after inhibitory avoidance training enhanced memory forty eight hours later (Zheng et al., 2008). It was also found that D1R antagonist infused into the amygdala or hippocampus after inhibitory avoidance training impaired memory when administered at certain time points (LaLumiere et al., 2004; Bernabeu et al., 1997; Bevilacqua et al., 1997; Rossato et al., 2009). DA infusions into the BLA within three hours of training enhanced retention when tested forty eight hours later whereas SCH 23390 administration resulted in impairment (LaLumiere et al., 2004). SCH 23390 was infused into the hippocampus immediately, nine or twelve hours after inhibitory avoidance training. It was found that SCH 23390 injected into the hippocampus twelve hours after training impaired memory seven and fourteen, but not two, days later whereas SCH 23390 injected immediately or nine hours after training had no effect (Rossato et al., 2009). Infusion of the D1R agonist SKF 38393 twelve hours after training, on the other hand, was found to increase memory seven and fourteen, but not two, days later (Rossato et al., 2009). SCH 23390 infused into the hippocampus but not the amygdala three or six hours, but not immediately after inhibitory avoidance, impaired memory whereas SKF 38393 facilitated memory formation

(Bernabeu et al., 1997; Bevilaqua et al., 1997). SCH 23390 administered into the CA1 immediately after inhibitory avoidance training did not affect LTM (24 hr) but enhanced STM (1.5 hr). Administration into the EC on the other hand, impaired LTM but not STM. SKF 38393 into the CA1 and EC impaired STM but not LTM (Izquierdo et al., 1998). Studies investigating contextual and cued fear memory consolidation however, have reported mixed results. Amphetamine infusion into the dHC immediately after auditory fear conditioning was found to increase freezing (i.e. improved memory) to background context but not to tone presentation (White and Salinas, 2003). SCH 23390 infused into the CA1 immediately after CFC, attenuated the enhancement in CFC brought about by systemic corticosterone, indicating that D1R are involved in modulating the actions of glucocorticoids in the hippocampus (Liao et al., 2013). Administration of SCH 23390 immediately after CFC had no effect (Inoue et al., 2000; Bai et al., 2009). These studies are outlined in chapter three in more detail.

D1Rs have also been proposed to be important in the synthesis of PRPs as outlined by the synaptic tagging and capture hypothesis (see above). It has been proposed that D1Rs are involved in the synthesis of PRPs (Wang et al., 2010; Sajj Kumar and Frey, 2004). Protein kinase M ζ , an isoform of PKC, has been proposed to be a PRP that is induced by DA activity (Navakkode et al., 2010). In another study, L-LTP was induced in one synapse S1 in hippocampal slices. SCH 23390 was then applied followed by stimulation of a second synapse S2. L-LTP occurred normally in both S1 and S2, indicating that D1R activated proteins whose synthesis was initiated by S1 stimulation can be used for L-LTP at S2 (Sajj Kumar and Frey, 2004). It has also been shown that L-LTP inducing stimulation of one pathway in the presence of SCH 23390 (which would normally impair it) was rescued by stimulation of a different pathway prior to SCH 23390 application (Wang et al., 2010). In the behaving animal it was found that novel exploration, which has been shown to result in increased DA release, prior to episodic-like memory formation in the presence of SCH 23390 can rescue that memory (Wang et al., 2010).

1.4.8 Dopaminergic Modulation of Retrieval

Studies investigating the involvement of DA in the retrieval of memory have produced mixed results. In one study rabbits underwent auditory fear conditioning where presentation of a tone (CS⁺) was associated with a shock to the pinna. Recordings from DA neurons in the VTA of rabbits showed that the majority of these neurons increased in activity following CS⁺ presentation whereas other neurons decreased their activity (Guarraci and Kapp, 1999). Intra-mPFC infusion of cis-flupenthixol (D1/D2R antagonist) was found to impair the retrieval of auditory fear (Pezze et al., 2003). In another study electrical stimulation of the VTA was found to increase FPS amplitudes (Borowski and Kokkinidis, 1996). However, intra-VTA administration of SCH 23390 or SKF 38393 prior to FPS testing was not found to affect retrieval (De Oliveira et al., 2009). Likewise systemic or intra-amygdala infusion of SCH 23390 had no effect (Greba and Kokkinidis, 2000). Infusions of SCH 23390 or SKF 38393 into the hippocampal formation had no effect on retrieval of inhibitory avoidance (Izquierdo et al., 1998). However, in another study, CA1 and EC infusions of SCH 23390 impaired retention of inhibitory avoidance whereas SKF 38393 enhanced it but intra-amygdala infusions had no effect (Barros et al., 2001). D1Rs appear to be required for the retrieval of object recognition memory in some circumstances (Hotte et al., 2005). The D1R agonist SKF 81297 was administered systemically prior to testing of object recognition either fifteen minutes or four hours after training. It was found that at the fifteen minute delay SKF 81297 at high or low doses left the rats unable to recognise the familiar object and therefore exploration of both novel and familiar objects was similar compared with controls, who explored the novel object more. At the four hour delay however, rats given the high dose of SKF 81297 showed enhanced object recognition retrieval compared with the rats administered a low dose or saline (Hotte et al., 2005). Western blot analysis showed that phosphorylated DARPP-32 levels in the PFC were increased in SKF 81297 treated rats following the four hour but not the fifteen minute delay (Hotte et al., 2006). Other research has shown the involvement of the D1Rs in the retrieval of fear using a second order conditioning paradigm

(Nader and LeDoux, 1999). SCH 23390 was infused directly into the BLA prior to the CS1 being paired with the CS2 and this resulted in impairment of second order fear conditioning, indicating that D1Rs are needed for retrieval (Nader and LeDoux, 1999). D1R activation in the mPFC was found to be important in the retrieval of olfactory fear-conditioning (Lauzon et al., 2009). Systemic administration of SCH 23390 was not found to impair the retrieval of contextual fear (Inoue et al., 2000). However other research has shown that SCH 23390 infused into the shell sub-region of the nucleus accumbens and anterior cingulate cortex increased rather than decreased freezing during contextual fear retrieval (Albrechet-Souza et al., 2013) and the DA agonist amphetamine was found to impair the retrieval of auditory fear (Pezze et al., 2003).

1.4.9 Dopaminergic Modulation of Reconsolidation

There have not been many previous studies looking at the involvement of DA in the reconsolidation of fear memories. However object recognition, passive avoidance memory in chicks and PCA have been investigated with mixed findings. The effects of infusing SCH 23390 (D1R antagonist) and SKF 38393 (D1R agonist) into the PFC on the consolidation and reconsolidation of recognition memory was tested using habituated (low arousal) and non-habituated (high arousal) rats with low and high corticosteroid, and therefore DA levels, respectively (see above). It was found that the habituated group had impaired reconsolidation when infused with SKF 38393 but SCH 23390 infusion had no effect, whereas reconsolidation was impaired by both drugs in the non-habituated group (Maroun and Akirav, 2009). The effect of SCH 23390 on reconsolidation in day old chicks using the single-trial passive avoidance task was studied. This involved training the chicks with balls covered in the aversive tasting methyl anthranilate. The chicks were then injected with SCH 23390 five minutes before reactivation. Their memory was tested by assessing their subsequent pecking rate on a dry ball. It was found that memory was impaired, implying that D1Rs are critically involved in the reconsolidation of passive avoidance in chicks (Sherry et al., 2005).

Amphetamine however, was not found to have an effect on reconsolidation of PCA using a sucrose reward (Blaiss and Janak, 2007). In another study however, it was found that injecting amphetamine immediately after reactivation resulted in enhancement of morphine CPP, indicating DA and/or NA involvement (Blaiss and Janak, 2006).

1.3.10 Dopaminergic Modulation of Destabilization

Research undertaken using reward paradigms, has shown the involvement of dopamine neurons in the VTA in learning mediated by prediction error. Prediction error has been proposed to be required for memory destabilization (see above), indicating that DA could be important in the destabilization process. DA neurons in the midbrain, including the SN and VTA, of monkeys have been shown to respond in a bidirectional way to positive and negative reward prediction error. Positive prediction error leads to these neurons increasing their activity whereas negative prediction errors result in neuronal depression (Schultz, 2013). A linear relationship between the probability of a prediction error occurring was also found in these neurons (Fiorillo et al., 2003). Other research using fMRI has shown that human brain regions which have strong DA projections such as the putamen (part of the striatum) and nucleus accumbens are activated by reward prediction errors. In one study a delayed incentive task was used where a cue represented the probability of obtaining a monetary reward if the subjects completed a simple task correctly. It was found that nucleus accumbens activity was high during the maximum level of positive prediction error (e.g. when the participants won despite only being cued with a 25% probability of winning) and was lowest during the minimum level of negative prediction error (e.g. when the participants lost despite being cued with a 75% probability of winning) (Ablner et al., 2006). In another study human participants were trained to associate a light presentation with delivery of juice six seconds later. To test temporal prediction errors 'catch events' were introduced in which the juice reward was delayed to ten seconds after the light presentation. Therefore a positive prediction error was created at the ten second time point (as participants

received an unexpected reward) and a negative prediction error was created at the six second time point (as the participants expected to gain a reward but did not). It was found that activity in the left and, to a lesser extent, right putamen was increased during the positive prediction error and decreased during the negative prediction error (McClure et al., 2003). Bilateral ventral striatum activation was correlated with both positive and negative reward prediction errors. L-DOPA (DA metabolic precursor) was found to enhance the blood oxygen level dependent signal compared with haloperidol (D2R antagonist), for the positive but not the negative prediction error (Pessiglione et al., 2006). Other research has used the Pavlovian over-expectation method in rats (Takahashi et al., 2009). In this task the rats first learnt to associate two auditory cues and one visual cue with delivery of three sucrose pellets into a food cup. The rats then underwent compound conditioning in which the visual cue and one of the auditory cues were paired together, resulting in delivery of three sucrose pellets for both cues rather than for each cue individually that the rats had been used to receiving. A negative prediction error was therefore created. After this the rats were tested for the amount of time they placed their noses in the food cap following presentation of all the cues individually. It was found that the rats spent less time waiting for the cues that had been compounded, indicating that they had learnt that these cues were no longer rewarded as well as the others. Inactivation of DA neurons in the VTA prior to compound conditioning resulted in these rats not altering their response to the less well rewarded cues during testing, indicating that the VTA is important for learning following negative prediction error (Takahashi et al., 2009). In another study using an appetitive goal-tracking paradigm it was found that a mix of muscimol and baclofen (GABA_B receptor agonist) infused into the VTA prior to reactivation prevented the normally amnesic effect of systemic MK-801 when administered after reactivation (Reichelt et al., 2013). This indicated the involvement of VTA neuronal activity in destabilization following negative prediction error. The VTA however, was not thought to be the site of this destabilization as intra-VTA APV or Mk-801 prior to reactivation did not mimic the effects of systemic

MK-801 administration and impair memory reconsolidation, implying that other brain regions are involved in the process (Reichelt et al., 2013).

1.3.11 Dopaminergic Modulation of Extinction

Researchers have started to investigate the role of DA in associative memory extinction. These studies have utilized dopamine agonists and antagonists and genetic knockout mice and have yielded mixed results. In one study methylphenidate was administered systemically prior to or immediately after contextual fear extinction training. It was found to improve subsequent retention (Abraham et al., 2012). Systemic administration of the DA agonist's amphetamine or cocaine or the D1R agonist SKF 38393 prior to FPS extinction training blocked extinction, leading to fear enhancement (Borowski and Kokkinidis, 1998). D1R KO mice were found to have impaired extinction of passive avoidance and contextual fear (EL-Ghundi et al., 2001). Auditory fear extinction was impaired by intra-PFC, but not intra-BLA, infusion of SCH 23390 (Hikind and Maroun, 2008). Intra-CA1 infusion of SCH 23390 impaired and SKF 38393 enhanced the extinction of both inhibitory avoidance and contextual fear (Fiorenza et al., 2012). Intra-BLA and intra-mPFC infusions of SCH 23390 and SKF 238393 on the other hand, had no effect on contextual fear extinction. Intra-BLA and intra-mPFC SCH 23390 impaired the extinction of inhibitory avoidance but SKF 38393 had no effect (Fiorenza et al., 2012). Extinction of CPP using cocaine was also found to depend on D1Rs (Fricks-Gleason et al., 2012). Systemic SCH 23390 was administered immediately following subsequent unreinforced extinction sessions. It was found that SCH 23390 increased the time for extinction to occur (i.e. no preference for the drug associated compartment), indicating the involvement of D1Rs in the extinction of appetitive memory (Fricks-Gleason et al., 2012). However, other studies have failed to find an involvement of DA or D1Rs in fear memory extinction. Systemic administration of amphetamine at high or low doses was not found to enhance extinction of auditory fear memory (Mueller et al., 2009; Carmack et al., 2010) and D1R KO mice were not found to have impaired extinction of contextual or auditory fear (Ortiz et al., 2010). In the

reward literature negative prediction error following reward omission has been proposed to result in extinction learning (Schultz, 2000). This has been shown in a study where rats that had previously been trained to associate a tone with delivery of sucrose, underwent extinction training which involved sucrose omission. A negative prediction error was therefore created. It was found that if DA neurons within the VTA were optogenetically activated during this prediction error (e.g. when these neurons would normally decrease their firing rates) then extinction learning was reduced (Steinberg et al., 2013).

1.3.12 Dopaminergic Modulation of Anxiety Disorders

The involvement of DA in anxiety disorders is starting to be investigated. It was found that the SLC6A3 3' variable number tandem repeat polymorphism of the DAT gene, which is linked to increased DA levels, was higher in PTSD compared with trauma-exposed non-PTSD controls (Segman et al., 2002). Plasma DA levels were also found to be increased in PTSD (Hamner and Diamond, 1993). In another study, homozygotes for the Val allele (Val/Val) of the val158met polymorphism of the COMT enzyme, which is associated with greater COMT activity, showed greater reacquisition (fear conditioning conducted after extinction has occurred) when extinction training was conducted six hours, but not ten minutes, after reactivation (Agren et al., 2012).

1.5 Aims and Objectives

The aims for the work carried out in this thesis were to investigate the role of D1Rs in the different learning and memory stages of contextual fear. The D1R selective antagonist SCH 23390 was therefore used. In the first set of experiments outlined in chapters three and four, the effects of systemic (chapter three) and central (chapter four) administration of SCH 23390 on acquisition and consolidation was investigated. It has already been demonstrated that systemic SCH 23390 impairs acquisition but not the consolidation of contextual fear (Inoue et al., 2000). The experiments undertaken in chapter three replicated this work. Previous research has shown that intra-dHC SCH 23390 impairs the acquisition of other types of memory, such as spatial (O'Carroll et al., 2006) and episodic-like (Bethus et al., 2010), however working memory in the radial maze was not impaired (Wilkerson and Levin, 1999). Intra-BLA infusion of the D1R agonist SKF 82958 was found to enhance the acquisition of contextual fear (Biendenkapp and Rudy, 2009), whereas it was found that intra- BLA SCH 23390 impaired the acquisition of FPS in one study (Greba and Kokkinidis, 2000) but not another (De Oliveira et al., 2011). It has not previously been shown if central administration of SCH 23390 into the BLA or dHC impairs the acquisition of contextual fear. The experiments undertaken in chapter four therefore extend previous research by administering SCH 23390 into either the dHC or BLA prior to CFC.

In the second set of experiments, outlined in chapters five and six, the effects of systemic SCH 23390 on contextual fear retrieval, reconsolidation, extinction and destabilization are investigated. Previous systemic research has shown that SCH 23390 did not impair contextual fear retrieval (Inoue et al., 2000). The reconsolidation of passive avoidance was impaired by systemic SCH 23390 administration (Sherry et al., 2005). Systemic administration of the non-selective DA agonist methylphenidate before and after contextual fear was found to improved auditory fear extinction (Abraham et al., 2012) whereas amphetamine did not (Mueller et al., 2009; Carmack et al., 2010) and

systemic SCH 23390 impaired the extinction of CPP (Fricks-Gleason et al., 2012). The experiments in chapters five therefore repeat previous research by testing the effect of systemic SCH 23390 prior to the retrieval of contextual fear. They also extend previous research by testing the effect of systemic SCH 23390 administration prior to and immediately after reconsolidation and extinction sessions. In chapter six, the effect of SCH 23390 on the destabilization of contextual fear was investigated as this has not been tested before.

Chapter Two

General Methods and Validation Studies

2.1 General methods

The general methods used for the experiments discussed in this thesis have been outlined below. Validation studies were carried out to determine the CFC parameters to be used. The first validation study determined the most suitable number of footshocks to use. The second and third validation studies investigated the effect of systemic NMDAR antagonism on CFC and its reconsolidation.

2.1.1 Animals

Male Lister hooded rats (Harlan, UK), weighing 250-400g were housed in groups of four on arrival to the animal unit and kept on a twelve hour light/dark cycle (lights on at 6:30am) with ad libitum food (standard chow) and water. They were habituated to handling (three days for two minutes each) before experiments were undertaken. The rats that were implanted with guide cannulae (see chapter 4) were housed separately after surgery and allowed at least five days to recover prior to behavioural testing. All methods used were conducted in accordance with the Animals (Scientific Procedures) Act 1986. A total of three hundred and twenty animals were used for the experiments carried out in this thesis.

2.1.2 Drugs

For systemic administration studies, SCH 23390 (Tocris, UK) and MK-801 (Sigma-Aldrich, UK) were made up fresh on the morning of use to the required concentration (0.1mg/kg) by dissolving in sterile saline (0.9%; 1 ml/kg). These doses of SCH 23390 and MK-801 have previously been shown to impair fear conditioning or fear memory reconsolidation (Inoue et al., 2000; Lee et al., 2006). SCH 23390 or MK-801 was injected either thirty minutes before or immediately after conditioning, reactivation or extinction sessions (see below). Controls received saline injections. All systemic drug administration experiments in this thesis used i.p. injections. Details on central drug infusions are presented in chapter four.

2.1.3 Behavioural Procedures

Contextual fear conditioning:

Fear conditioning took place in two chambers from Med Associates (base: 30 x 24 cm, height: 30 cm) connected to an electric shock generator which delivered constant current shocks via the metal grid floor. A speaker and light were mounted on the side of the chamber. The light came on at the beginning of the conditioning and memory testing sessions and stayed on throughout, this added to the contextual background. White noise (60 dB) was also played throughout to lessen the impact of outside noise. The chambers were decorated with either stripes or spots and cleaned with 40% ethanol solution to provide a distinctive contextual background for the rats.

During conditioning the rats were individually placed into either one of the two chambers. In the first validation study, the rats waited two minutes before receiving either four or six unsignalled footshocks (one minute inter-trial interval (ITI)) and were removed two minutes after the last shock (see below). In all subsequent experiments the rats received four footshocks during conditioning. For the systemic drug injection experiments, the footshocks were half a mA and lasted for half a second. The duration of the footshocks was increased to one second for the central drug infusion experiments because rats with implants are less sensitive to footshock administration. For the acquisition and consolidation experiments, the rats were returned to the same chamber twenty four hours and seven days after conditioning for a two minute LTM retention test, during which their behaviour was digitally recorded. Videos were later analysed for freezing behaviour. Freezing behaviour (defined as no movement apart from that required for respiration) was scored manually by observation at three-second intervals and calculated as a percentage of the two minute test sessions. The animals were randomly assigned to groups and the scoring was carried out blind to condition by two observers.

For the reconsolidation and destabilization experiments, the rats were conditioned as above. However, they received a two minute reactivation session twenty four hours later, where they were returned to the same chamber before undergoing two minute PR-LTM retention tests twenty four hours and seven days after the reactivation session. Behaviour was digitally recorded during each session and freezing behaviour was assessed during the last two minutes of the conditioning session, the reactivation session and the twenty four hours and seven day PR-LTM retention sessions.

For the extinction experiments the rats were conditioned as above, however, they then underwent an extinction session twenty four hours later, where they were returned to the same chamber as conditioning for twenty minutes without receiving any footshocks. They then underwent retention testing twenty four hours and seven days after the extinction session. Behaviour was digitally recorded during each session and freezing behaviour was assessed during the last two minutes of the conditioning session, the first and last two minutes of the extinction session, and the twenty four hour and seven day retention sessions.

Open Field:

Some of the same rats that were fear conditioned were later tested in the open field (black perspex, base: 100 x 100 cm, enclosing wall: 50 cm high) to examine the effects of SCH 23390 on locomotor activity and innate fear. All rats were introduced into the apparatus at the same peripheral location and the testing was conducted in a dimly lit room for ten minutes. Behaviour was recorded with a video camera positioned directly above the arena for later analysis (see below). The rats that were given SCH 23390 or vehicle systemically were placed individually into the open field arena either thirty minutes or twenty four hours later. The rats that were infused with SCH 23390 or vehicle into the dHC or BLA were placed individually into the arena ten minutes after microinfusion (see below). Open field data were analysed

using Ethovision software and the following variables were quantified: total distance moved, rearing, and time spent in the centre.

Shock Sensitivity:

Some of the same rats that were fear conditioned were later assessed for their shock sensitivity threshold. The rats were injected with SCH 23390 or saline thirty minutes before being placed into a novel chamber and, after ten minutes of habituation, were administered ten footshocks (one minute ITI) increasing in intensity from 0.05-0.5 mA. Behaviour was recorded and the threshold for the first flinch (raising hind paws briefly off the bars) and first audible vocalization following footsock administration was assessed in each rat (Quick et al., 2000).

2.2 Validation Studies

The first validation study was carried out to assess the optimal number of footshocks to administer during conditioning in order to obtain moderate (i.e. 40-50%) freezing levels during subsequent memory retention tests; this was to avoid potential floor or ceiling effects. In the second validation study the NMDAR antagonist MK-801 was administered systemically (i.p.) prior to conditioning in order to assess the injection procedure and to replicate previous findings showing impaired conditioning with this treatment. In the third validation study MK-801 was administered systemically prior to reactivation to assess the effects of NMDAR antagonism on reconsolidation.

2.2.1 Four vs Six Footshocks on Contextual Fear Conditioning

Methods

The contextual fear apparatus and procedure have been outlined in detail in section 2.1. Half the rats (n=10) received six footshocks, whereas the other half (n=10) received four footshocks during conditioning. Two-way mixed analysis of variance (ANOVA) was used to analyse the freezing levels, with footshock (four or six) as the between-subject factor and memory testing

session (e.g. conditioning (COND), reactivation (REACT), 24 hr and 7 d) as the within-subject factor. All data are presented as the mean + standard error of the mean (SEM).

Results

Both six and four footshocks led to freezing behaviour after the last shock during the conditioning session, during reactivation (also equivalent to LTM testing 24hrs after conditioning), and during PR-LTM testing twenty four hours and seven days after reactivation (see Fig 2.1). A two-way mixed ANOVA revealed no main effect of footshock ($F_{(1,18)}=0.393$, $P>0.05$) and no footshock x time interaction ($F_{(3,54)}=0.292$, $P>0.05$). Both six and four footshocks resulted in freezing behaviour of ~80% in the last two minutes of the conditioning session and for the reactivation session 46% and 54% respectively. Six footshocks resulted in freezing levels of around 30% during the twenty four hour and seven day PR-LTM retention sessions, whereas four footshocks resulted in freezing levels of 38% and 36% during the twenty four hour and seven day PR-LTM retention sessions respectively.

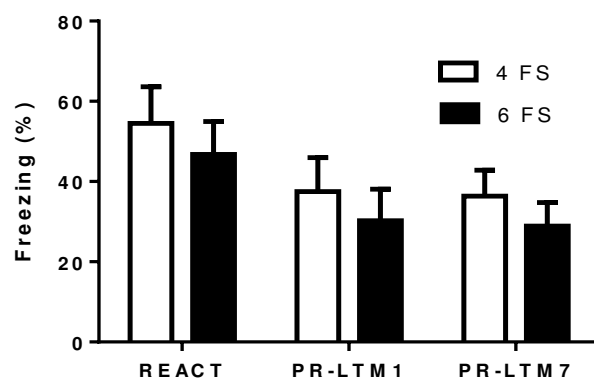


Fig 2.1 The effect of six vs four footshocks (FS). There were no differences in freezing between six and four FS during contextual fear reactivation (REACT), PR-LTM 24 hr after reactivation (PR-LTM1) and PR-LTM 7 d after reactivation (PR-LTM7)

Discussion

An optimum freezing level of 40-50% during reactivation (i.e. LTM testing) was achieved by administering four footshocks during the conditioning session, freezing did not differ significantly between rats conditioned using four or six shocks. To minimise unnecessary suffering experienced by the rats and

because strong conditioning has previously been shown to be more resistant to undergoing reconsolidation (Suzuki et al., 2004), the experiments in this thesis were therefore all conducted using a four footshock CFC protocol.

2.2.2 Systemic NMDA Receptor Antagonism during Contextual Fear Conditioning

Introduction

The second validation study was undertaken to assess the systemic injection procedure and to determine if conditioning using the four footshock protocol could be effectively impaired by NMDAR antagonism. Previous research has shown that NMDA receptor antagonists impair many different types of memory acquisition. For example, systemic injection of MK-801 was found to impair the acquisition of tasks in the Morris water maze, T-maze, passive avoidance and object recognition (van de Staays et al., 2011) and CFC (Gould et al., 2002). MK-801 or vehicle was injected thirty minutes prior to CFC.

Methods

The methods for this experiment are outlined in detail in section 2.1. Briefly, MK-801 (n=8) or vehicle (n=7) was injected i.p. thirty minutes prior to CFC. Two minute LTM retention sessions were conducted twenty four hours and seven days later, during which freezing behaviour was assessed. Two-way mixed ANOVA was used to analyse the freezing levels, with drug (MK-801 or vehicle) as the between-subject factor and memory testing session (e.g. COND, 24 hr and 7 d) as the within-subject factor. All data are presented as the mean + SEM.

Results

It was found that, compared to vehicle, systemic MK-801 given prior to CFC impaired freezing during the last two minutes of the conditioning session and during subsequent memory retention sessions twenty four hours and seven days later (See Fig 2.2). Analysis of freezing behaviour using a two-way

ANOVA revealed a significant main effect of drug ($F_{1,13}=5.974$, $P<0.05$) but no drug x time interaction ($F_{2,26}=1.369$, $P>0.05$).

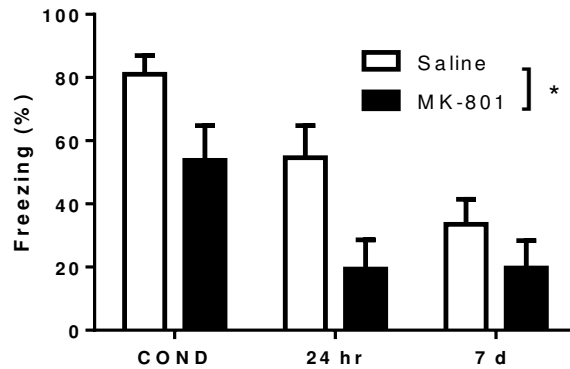


Fig 2.2 The effect of NMDA receptor antagonism on contextual fear conditioning. MK-801 prior to conditioning decreased freezing in the last two minutes of the conditioning session (COND) and during the retention sessions (* $P<0.05$)

Discussion

It was found that systemic NMDA receptor antagonism impaired the acquisition of contextual fear. MK-801 given prior to conditioning led to reduced freezing at the end of the conditioning session and during the LTM retention sessions twenty four hours and seven days later. This is in agreement with a previous study showing that systemic and intra-hippocampal MK-801 prior to conditioning impaired contextual but not auditory fear (Gould et al., 2002; Bast et al., 2003). Other studies using the NMDA receptor antagonist APV were also found to impair CFC with intra-ventricular (Kim et al., 1991) intra-amygdala (Maren et al., 1996b) and intra-hippocampal (Young et al., 1994) infusion. This study demonstrates that the injection procedure was carried out correctly and that the conditioning procedure using four footshocks gives an optimum level of freezing behaviour that is able to be reduced by NMDAR antagonism.

2.2.3 Systemic NMDA Receptor Antagonism during Contextual Fear Reactivation

Introduction

The third validation study was undertaken to determine if the conditioning paradigm allows for pharmacological disruption of reconsolidation. Previous research has shown that NMDAR antagonists impair reconsolidation. For example, systemic injection of MK-801 was found to impair auditory fear reconsolidation (Lee et al., 2006). MK-801 or vehicle was injected thirty minutes prior to contextual fear memory reactivation. To the best of our knowledge this has not been tested before, for contextual fear.

Methods

The methods for this experiment are outlined in detail in section 2.1. Briefly, MK-801 (n=9) or vehicle (n=9) was injected i.p. thirty minutes prior to contextual fear reactivation. Two minute PR-LTM retention sessions were conducted twenty four hours (PR-LTM1) and seven days (PR-LTM7) later, during which freezing behaviour was assessed. Two-way mixed ANOVA was used to analyse the freezing levels, with drug (MK-801 or vehicle) as the between-subject factor and memory testing session (e.g. COND, REACT, PR-LTM1 and PR-LTM7) as the within-subject factor. All data are presented as the mean + SEM.

Results

It was found that, compared to vehicle, systemic MK-801 given prior to contextual fear reactivation impaired freezing behaviour (see Fig 2.3). Analysis of freezing behaviour using a two-way ANOVA revealed a main effect of drug ($F_{(1,15)}=5.747$, $P<0.05$) and a drug x time interaction ($F_{(3,45)}=3.364$, $P<0.05$). Post hoc analysis using independent t-tests indicated that freezing behaviour was significantly decreased during the reactivation session and PR-LTM1 twenty four hours later ($P<0.05$). As only three groups were used and a significant F value was obtained the max type one error rate is held at the

chosen level of 5% and therefore the post hoc analysis does not need to be corrected for repeated measures (Cardinal and Aitken, 2006).

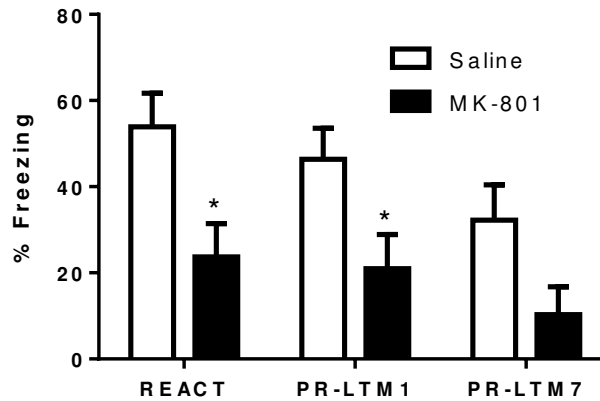


Fig 2.3 The effect of NMDA receptor antagonism on contextual fear reconsolidation. MK-801 prior to reactivation impaired freezing levels during reactivation (REACT) and PR-LTM 24 hr (PR-LTM1) but not 7 d (PR-LTM7) later (*P<0.05)

Discussion

Systemic administration of MK-801 prior to reactivation was found to impair contextual fear reconsolidation. Freezing behaviour was reduced in the drug group during post reactivation testing sessions twenty four hours later. This has, to the best of our knowledge, not previously been demonstrated and indicates the involvement of NMDARs in the reconsolidation of contextual fear. This finding is in agreement with research undertaken using other memory paradigms. Systemic MK-801 prior to reactivation impaired reconsolidation of auditory fear (Lee et al., 2006), appetitive memory (Lee and Everitt, 2008) and drug seeking associative memory (Milton et al., 2008). In another study systemic MK-801 prior to reactivation of an appetitive goal tracking task was found to impair PR-LTM if six, but not twelve or three days of training were given (Reichelt and Lee, 2012). This result indicates that the CFC procedure used in our experiment was not too strong and therefore able to undergo reconsolidation and also that a reactivation session of two minutes was adequate for reconsolidation to occur.

Chapter Two Summary

The effect of administering four vs six footshocks was tested in order to determine what CFC protocol to use for the experiments undertaken in this thesis. Both four and six footshocks led to optimal freezing levels of around 50% during the reactivation session, therefore in order to minimise rat suffering, a four footshock protocol was used for all subsequent experiments outlined in this thesis. The effect of systemic NMDAR antagonism on contextual fear was also investigated. It was found that MK-801 administered prior to CFC impaired its acquisition in agreement with previous research. It was also found that MK-801 administration prior to contextual fear reactivation impaired reconsolidation. To the best of our knowledge this has not previously been demonstrated. These results validate the i.p. injection procedure, the MK-801 dose and the contextual fear behavioural parameters used in the experiments contained within this thesis.

Chapter Three

**Systemic D1 Receptor Antagonism during
Contextual Fear Acquisition and
Consolidation**

3.1 Introduction

Activation of D1Rs has been shown to lead to the initiation of a number of molecular pathways that are thought to be involved in initial fear memory formation. D1R stimulation results in the activation of adenylate cyclase which leads to increased levels of cAMP which, in turn, activates PKA. It was found that intraventricular infusion of PKA inhibitors impaired contextual fear memory consolidation (Schafe et al., 1999). Both the cAMP/PKA and PLC pathways culminate in the activation of the transcription factor CREB, which can initiate synthesis of new proteins and lead to modulation of ion channels. It has been shown that CREB KO mice also have impaired CFC (Bourtchouladze et al., 1994). Other evidence for the involvement of DA in fear memory acquisition has also been obtained using transgenic mice. D1R KO mice have been shown to have reduced contextual and cued fear conditioning (Ortiz et al., 2010), and DA deficient and D1R KO mice were found to have impaired inhibitory avoidance memory (Fadok et al., 2009).

Previous research using DA, and D1R antagonists and agonists, has also been undertaken. In one study withdrawal from chronic amphetamine prior to conditioning was found to enhance memory formation; freezing behaviour in amphetamine pre-treated rats was increased during conditioning and subsequent tone alone memory testing sessions (Pezze et al., 2002). Another DA agonist, apomorphine, was found to enhance two-way avoidance learning as apomorphine injected rats learnt faster than saline injected controls (Reis et al., 2004). Other studies have made use of the selective D1R antagonist SCH 23390. Systemic SCH 23390 prior to fear training has been found to impair FPS (Greba and Kokkinidis, 2000) and two-way avoidance learning (Reis et al., 2004). It has been previously demonstrated that systemic administration of SCH 23390 given before CFC impairs freezing behaviour twenty four hours later (Inoue et al., 2000; Calzavara et al., 2009), whereas SCH 23390 administered immediately after CFC does not impair freezing behaviour twenty four hours later (Inoue et al., 2000; Bai et al., 2009).

Chapter Three Aims

The experiments in this chapter re-examine previous research looking at the effects of systemic SCH 23390 administrations either before or immediately after CFC in rats. To eliminate the possibility that the effect of SCH 23390 on fear learning was a result of non-specific effects on locomotion or pain sensitivity, the rats were also tested in the open field thirty minutes and twenty four hours after drug administration and had their shock sensitivity threshold assessed.

3.2 Methods

The methods used in this chapter have been outlined in chapter two. Briefly, in the CFC experiments, SCH 23390 or saline was injected either thirty minutes before (SCH 23390 n=10, saline n=10) or immediately after (SCH 23390 n=10, saline n=10) CFC (four 0.5mA, half second footshocks; one minute ITI). The rats underwent a two minute retention test twenty four hours and seven days after conditioning. Freezing behaviour at the end of conditioning and during LTM testing was subsequently scored. In the open field experiments, SCH 23390 or saline was injected either thirty minutes (SCH 23390 n=11, saline n=9) or twenty four hours (SCH 23390 n=9, saline n=9) prior to placement in the open field arena for ten minutes. The total distance moved and time spent in the inner-zone of the arena for each rat was obtained using Ethovision software. Rearing behaviour was assessed manually by the experimenter at the time of testing. In the shock sensitivity threshold experiment, rats were injected with SCH 23390 (n=10) or saline (n=10) thirty minutes prior to placement in novel chambers, where they received ten footshocks increasing in intensity from 0.05-0.5 mA. Behaviour was recorded and the rats' thresholds to flinch and to vocalize were both assessed.

Two-way mixed ANOVAs were used to analyse the freezing levels, with drug (SCH 23390 or vehicle) as the between-subject factor and memory testing session (24 hr and 7 d) as the within-subject factor in the CFC experiments. In the pain sensitivity experiment drug was the between-subject factor (SCH

23390 or vehicle) and shock intensity was the within-subject factor (flinch and vocalize). Open field data were analysed using Ethovision software. Unpaired t-tests were used to compare the total distance moved, the rearing frequency (defined as lifting of front paws into the air or onto the wall of the arena) and percentage of time spent in the centre of the open field between the drug and saline treated groups. All data are presented as the mean + SEM.

3.3 Results

3.3.1 Effect of Systemic D1 Receptor Antagonism on Contextual Fear

Administration of SCH 23390 thirty minutes before CFC impaired freezing behaviour twenty four hours later (see Fig 3.1). Analysis of freezing behaviour when drug was given before conditioning using a two-way ANOVA revealed a significant main effect of drug ($F_{(1,18)}=5.578$, $P<0.05$) and drug x time interaction ($F_{(1,18)}=14.039$, $P<0.01$). Post hoc analysis using an independent t-test indicated that freezing behaviour was significantly decreased during LTM testing twenty four hours later ($P<0.01$). As SCH 23390 was found to effect behaviour in the open field when administered thirty minutes prior to testing (see below), the data for freezing behaviour during the last two minutes of the conditioning session were not included in the analysis as non-specific effects of the drug could not be ruled out.

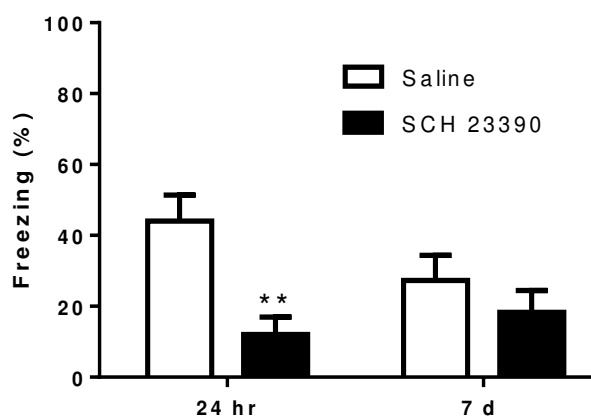


Fig 3.1 The effect of D1R antagonism during contextual fear conditioning. SCH 23390 prior to conditioning decreased freezing during memory retention 24 hr later (** $P<0.01$).

Administration of SCH 23390 immediately after CFC had no effect on freezing behaviour during memory retention sessions twenty four hours and seven

days later (see Fig 3.2). Analysis revealed no significant main effects of drug ($F_{(1,18)}=0.224$, $P>0.05$) and no drug x time interaction ($F_{(2,36)}=0.808$, $P>0.05$).

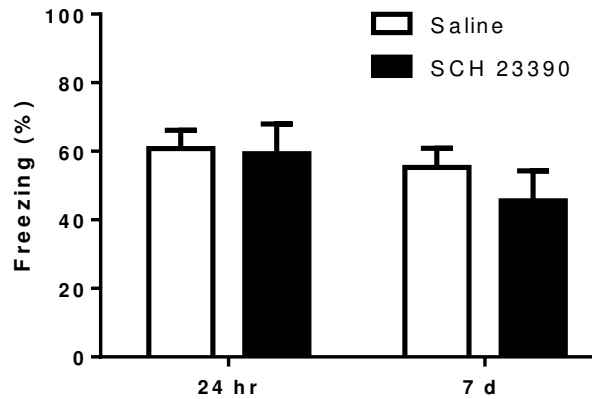


Fig 3.2 The effect of D1R antagonism after contextual fear conditioning. SCH 23390 immediately after conditioning did not alter freezing during memory retention sessions.

3.3.2 Effect of Systemic D1 Receptor Antagonism on Behaviour in the Open Field

To eliminate the possibility that the effects of SCH 23390 on CFC were due to non-specific effects of the drug on locomotion or innate fear, behaviour in the open field was assessed (see Fig 3.3 and 3.4). SCH 23390 administered systemically thirty minutes prior to open field testing reduced total distance moved ($t_{(12)}=4.04$, $P<0.01$), rearing frequency ($t_{(18)}=2.76$, $P<0.05$) and time spent in the inner-zone ($t_{(16)}=2.16$, $P<0.05$).

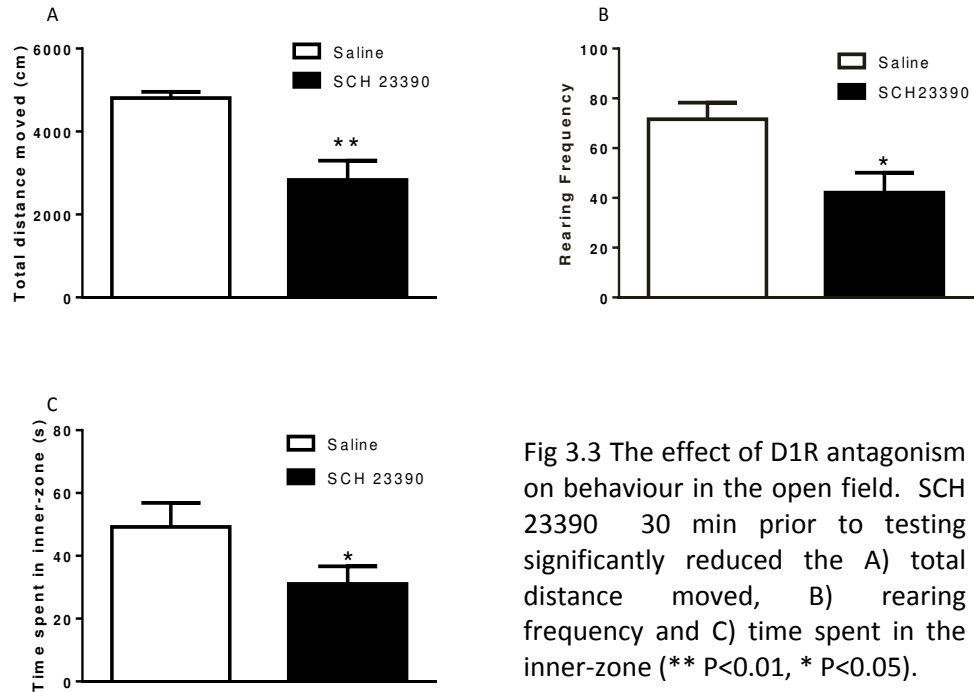


Fig 3.3 The effect of D1R antagonism on behaviour in the open field. SCH 23390 30 min prior to testing significantly reduced the A) total distance moved, B) rearing frequency and C) time spent in the inner-zone (** P<0.01, * P<0.05).

However, administration of SCH 23390 twenty four hours prior to open field testing did not affect total distance moved ($t_{(16)}=0.266$, $P>0.05$), rearing frequency ($t_{(16)}=0.478$, $P>0.05$) or time spent in the inner-zone ($t_{(16)}=0.484$, $P>0.05$).

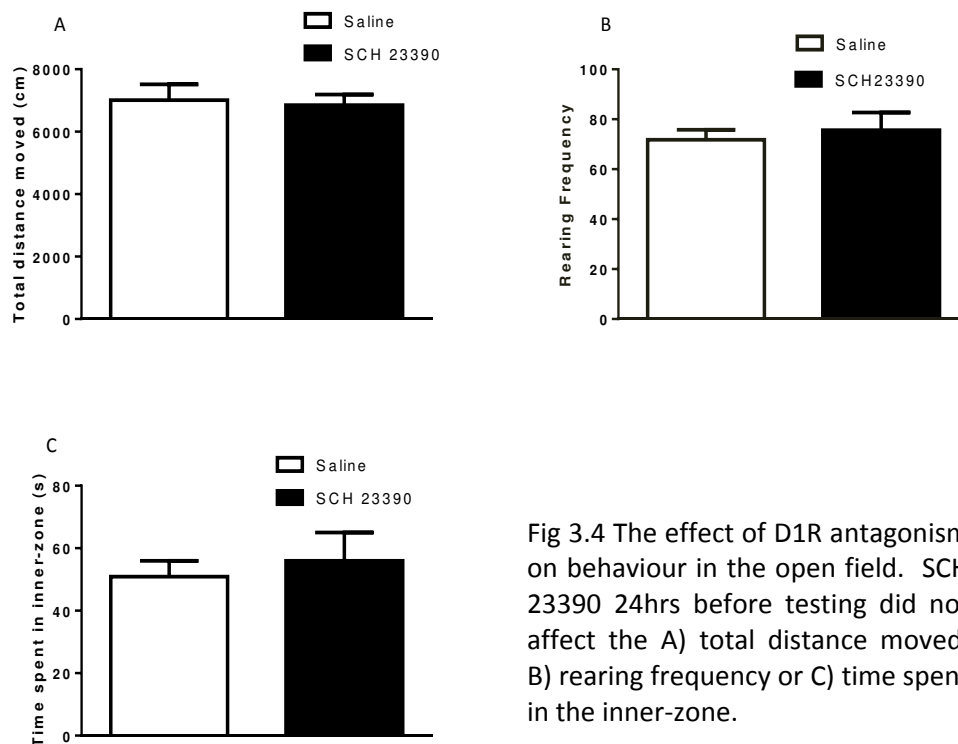


Fig 3.4 The effect of D1R antagonism on behaviour in the open field. SCH 23390 24hrs before testing did not affect the A) total distance moved, B) rearing frequency or C) time spent in the inner-zone.

3.3.3 Effect of Systemic D1 Receptor Antagonism on Pain Sensitivity

To eliminate the possibility that the effects of SCH 23390 on contextual fear were due to side effects of SCH 23390 on the pain experienced by the rats, their shock sensitivity threshold was assessed (see Fig 3.5). A two-way ANOVA revealed no significant main effect of drug on shock sensitivity threshold ($F_{(1,18)}=0.010$, $P>0.05$).

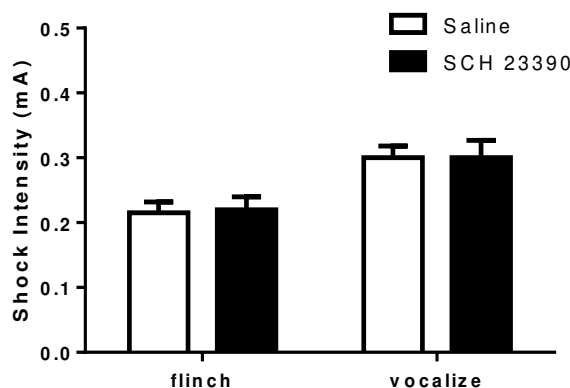


Fig 3.5 The effect of D1R antagonism on pain sensitivity. SCH 23390 did not alter the rats' threshold to flinch or vocalize in response to footshock.

3.4 Discussion

Systemic D1R antagonism during CFC was found to impair acquisition. This indicates that DA acting at the D1 receptor is required during the initial formation of contextual fear memories. In contrast SCH 23390 administered immediately after contextual fear memory had no effect on subsequent memory retention. Administration of SCH 23390 thirty minutes prior to open field testing reduced total distance moved, rearing frequency and the time spent in the inner-zone, whereas administration twenty four hours prior to open field testing had no effect on these behaviours. The shock sensitivity thresholds were similar between the drug and saline treated rats, indicating that SCH 23390 does not affect the pain experience of the footshock and thus the rats' ability to associate it with the fear conditioning chamber due to impaired nociception. This confirms previous findings indicating that D1R antagonism has no effect on shock sensitivity (Inoue et al., 2000).

3.4.1 Systemic D1 Receptor Antagonism during Acquisition

The finding that systemic administration of SCH 23390 impairs the acquisition of contextual fear when administered prior to CFC is in agreement with previous research (Inoue et al., 2000, Calzavara et al., 2009). Inoue and colleagues (2000) found that systemic SCH 23390 thirty minutes prior to CFC reduced freezing twenty four hours later (Inoue et al., 2000; Inoue et al., 2005). This reduction in freezing was found to be increased by administration of the D2R antagonist haloperidol at the same time, indicating D2R, as well as D1R involvement (Inoue et al., 2005). Systemic SCH 23390 fifteen minutes prior to conditioning was also found to reduce freezing twenty four hours later in both spontaneously hypertensive rats and normotensive Wistar rats. The D2R antagonist metoclopramide, on the other hand, did not affect freezing in either group (Calzavara et al., 2009). SCH 23390 also weakly antagonises 5-HT₂ receptors (Hyttel, 1983); however, it has been shown that the 5-HT₂ antagonist ICI169369 did not impair contextual fear acquisition, indicating that the effects of SCH 23390 on contextual fear acquisition result

from the antagonism of D1Rs and not 5-HT₂ receptors (Inoue et al., 1996). It can be difficult to determine if a drug given before conditioning is having an effect specifically on acquisition, short term or long term consolidation processes (Bast et al., 2001). However, because in our study freezing behaviour was impaired in the last two minutes of the conditioning session, and SCH 23390 given immediately after conditioning did not affect freezing levels, it is most likely that the acquisition, as opposed to short or long term consolidation processes, were affected by D1R antagonism. The shock sensitivity thresholds were similar between the drug and saline treated rats, indicating that the effects of SCH 23390 on the acquisition of contextual fear are a result of impaired memory acquisition rather than due to differences in the pain experienced by the rats.

There are mixed results on the effect of SCH 23390 given prior to training in other fear memory paradigms. For example, administration before conditioning to a light stimulus paired with foot shocks reduced freezing to the light stimulus alone but did not affect the FPS response (De Oliveira et al., 2006), whereas other research showed that SCH 23390 prior to fear conditioning did impair FPS (Greba and Kokkinidis, 2000). Systemic SCH 23390 prior to training was found to impair short term object recognition memory formation (Clausen et al., 2011). The conflicts with previous research are possibly due to the memory paradigms used. For example object recognition tasks may involve more complex cognitive functions as they measure the rats' choices (e.g. explore or do not explore an object). On the other hand, in CFC and retention testing the rats are placed into a conditioning chamber for a set period during which they cannot escape (i.e. they have no choice). Therefore these memory paradigms likely involve different neural pathways and/or neurochemical mechanisms that rely differently on local D1R activation.

The synaptic tagging and capture hypothesis postulates that during the induction of E-LTP active synapses are 'tagged' which allow them to later receive PRPs, enabling E-LTP to be converted to L-LTP (Frey and Morris, 1997).

It has been proposed that D1Rs are involved in the synthesis of PRPs (Wang et al., 2010; Sajikumar and Frey, 2004). It could be that SCH 23390 given prior to CFC impairs the initiation of PRP synthesis and so E-LTP is not being converted to late L-LTP. Therefore the rats do not remember the association between the fear conditioning chamber and the footshock twenty four hours later.

3.4.2 Systemic D1 Receptor Antagonism during Consolidation

The finding that systemic administration of SCH 23390 after CFC did not impair freezing behaviour twenty four hours or seven days later is in agreement with previous research (Inoue et al., 2000; Bai et al., 2009). It would therefore seem that D1R signalling is not involved in contextual fear consolidation. In one study, SCH 23390 injection thirty minutes after CFC was not found to impair freezing behaviour twenty four hours later (Inoue et al., 2000). In another SCH 23390 administered three times after CFC did not impair memory twenty four hours or eight days later (Bai et al., 2009). It has also been shown that while SCH 23390 application during high frequency stimulation in CA1 slice preparations resulted in reduced LTP, SCH 23390 application immediately after high frequency stimulation had no effect (Frey et al., 1991). One possible explanation for the lack of effect of D1R antagonism immediately after CFC could be that D1R activation had already initiated PRP synthesis by the time SCH 23390 was administered. That is, the mechanisms had already been set in motion and impairing D1R activity after CFC is too late to interfere with this.

3.4.3 Systemic D1 Receptor Antagonism and Behaviour in the Open Field

The finding that systemic SCH 23390 administered thirty minutes prior to open field testing significantly reduced total distance moved and rearing frequency is in agreement with previous research (Gessa et al., 1985; Salmi and Ahlenius, 2000; Clausen et al., 2011). SCH 23390 has also been shown to reduce locomotion induced by D2R agonists such as the non-selective D2R agonist apomorphine or the selective D2R agonist Ru 24213 (Molloy et al., 1986). It has been proposed that high doses of SCH 23390 reduce locomotion

and rearing behaviour but that low doses can increase locomotion (Bruhwylter et al., 1991). SCH 23390 administered thirty minutes prior to testing for ten minutes in the open field decreased peripheral and central ambulation at high doses (0.1 and 0.3mg/kg) but increased them at low doses (0.01 and 0.03 mg/kg) (Bruhwylter et al., 1991). However another study found that a dose of 0.1mg/kg did not reduce locomotion (De Oliveira et al., 2006). SCH 23390 administered twenty four hours before open field testing did not affect total distance moved, rearing frequency or time spent in the inner-zone of the arena. This finding indicates that SCH 23390 is no longer affecting the rats twenty four hours later when they undergo contextual fear memory testing, in agreement with the short half-life (thirty minutes) of this drug (Andersen and Gronvald, 1986). The reduction in freezing behaviour observed during the twenty four hour and seven day memory retention sessions was therefore unlikely to be a consequence of non-specific effects of SCH 23390 on locomotion. Given that SCH 23390 reduced the total distance moved in the open field it is difficult to draw conclusions on the time spent in the inner-zone on, for example, drug induced increases in anxiety as the reduction could be a result of reduced locomotion overall.

3.4.4 Chapter Three Summary

The effect of systemic D1R antagonism on contextual fear was investigated. It was found that SCH 23390 administered prior to CFC impaired acquisition as memory retention was significantly impaired twenty four hours later. On the other hand SCH 23390 administered immediately after conditioning had no effect. These results are in agreement with previous research. This impairment of CFC acquisition by D1R antagonism is unlikely to be a result of non-specific effects of the drug on locomotion as administration of SCH 23390 twenty four hours prior to open field testing did not affect total distance moved. It is also unlikely to be a result of non-specific effects of the drug on nociception as both SCH 23390 and saline injected rats flinched and vocalized at the same level of footshock administration. The experiments outlined in

chapter four extend these findings by exploring where in the brain this effect of D1R antagonism on contextual fear could be taking place.

Chapter Four

**D1 Receptor Antagonism in the
Hippocampus and Amygdala during
Contextual Fear Acquisition**

4.1 Introduction

The experiments outlined in chapter three showed that systemic administration of SCH 23390 prior to conditioning impairs the acquisition of contextual fear. The logical next stage was therefore to try to determine which brain regions were mediating this effect. The dHc and BLA nucleus of the amygdala were chosen as previous research has linked these brain regions with DA modulation of fear learning. Evidence for involvement of the dHc and BLA in fear memory acquisition has been demonstrated using lesion methods. Pre-training lesions of both the dHc (Kim et al., 1993; Young et al., 1994) and BLA (Kim et al., 1993; Phillips and LeDoux, 1992) have been found to impair freezing to contextual stimuli. These studies indicate that these regions are important in contextual fear acquisition under normal circumstances (Fanselow, 2010). The hippocampus and amygdala receive dopaminergic projections from the SN-VTA forming part of the mesolimbic DA pathway. Injections of [³H] leucine (an anterograde tracer) and horseradish peroxidase (a retrograde tracer) into VTA showed projections to the amygdala including to the BLA (Simon et al., 1979). Retrograde tracing using fluorescent tracers such as fluoro-gold and immunocytochemistry staining for TH showed that dopaminergic neurons from the VTA project to the hippocampal formation (Gasbarri et al., 1994). D1R are expressed in both the hippocampus and amygdala (Boyson et al., 1986; Weiner et al., 1991; Huang et al., 1992).

Previous research has shown a link between memory acquisition and DA modulation in the amygdala and hippocampus. DA levels in the hippocampus and DOPAC levels in the amygdala were found to be elevated following footshock administration (Inoue et al., 1994). LTP is a proposed cellular model of memory formation and has been demonstrated in both the hippocampus (Bliss and Lomo, 1973) and amygdala (Rogan et al., 1997). D1R antagonism has been shown to impair both E-LTP (Otmakhova and Lisman, 1996) and L-LTP (Huang and Kandel, 1995) in the hippocampus. Studies using D1R agonists and antagonists have investigated the involvement of DA in

numerous memory paradigms, including spatial (O'Carroll et al., 2006; Pezze and Bast, 2012) and episodic-like memory (Bethus et al., 2010). The involvement of amygdala and hippocampal DA modulation of inhibitory avoidance has also been investigated. DA deficient mice were found to have impaired inhibitory avoidance memory and this could be rescued by restoration of DA levels by L-DOPA administration in the amygdala (Fadok et al., 2009). There have also been studies undertaken looking at the involvement of D1Rs in the acquisition and consolidation of Pavlovian fear memory. Intra-BLA infusion of SCH 23390 prior to training was found to impair FPS acquisition (Greba and Kokkinidis, 2000). SCH 23390 and SKF 82958 (D1 agonist) infused prior to auditory training into the cAMY prior to auditory training resulted in freezing impairment and enhancement respectively, to both background context and tone presentations twenty four hours later (Guarraci et al., 1999). Infusion of SKF 82958 into the BLA was found to enhance CFC (Biedenkapp and Rudy, 2009).

Chapter Four Aims

The aim of the experiments in this chapter was to determine if the impairment in the acquisition of contextual fear resulting from systemic D1R antagonism could be taking place in either the dHC or BLA. Although the dHC and BLA are necessary under normal circumstances for contextual fear acquisition, the role of DA in this process remains unclear. Therefore the effects of intra-dHC and intra-BLA SCH 23390 administration prior to CFC were examined. To eliminate the possibility that the effect of SCH 23390 on fear learning could result from non-specific effects on locomotion, the rats were also tested in the open field following intra-dHC and intra-BLA SCH 23390 infusions.

4.2 Methods

4.2.1 Surgery

Sixty two rats were habituated to the animal unit for one week before being implanted with bilateral guide cannulae (26 gauge) and stylets (33 gauge)

aimed at the dHC or BLA. Anaesthesia was induced with 4-4.5% isoflurane in air and maintained on 1-3%. They were placed in a stereotaxic frame and implanted with bilateral cannulae aimed at the following coordinates: dHC: 4.5 mm posterior and 3 mm lateral from bregma, 3 mm ventral to the brain surface; BLA: 2.8 mm posterior and 4.7 mm lateral from bregma, 6.4 mm ventral to the brain surface. The cannulae were secured to the skull with four stainless steel screws and dental cement. For analgesia, the rats were injected with buprenorphine (0.05mg/kg) at the start of surgery, between six to eight hours later and the following morning; they were also injected with meloxicam (2mg/kg) at the end of surgery and received it orally for four days post surgery. They were allowed to recover for at least five days before undergoing behavioural testing, during which time they were habituated for the drug microinfusions by gentle restraint followed by removal and reinsertion of their cannulae stylets.

4.2.2 Drugs

SCH 23390 was made up to a concentration of 5µg/µL in sterile saline. This concentration of SCH 23390 has been used previously to examine the role of hippocampal D1R signalling in spatial memory processing (O'Carroll et al., 2006). In the dHC, SCH 23390 (5µg in 1 µL per side) was infused over a two minute period. In BLA, SCH 23390 (2.5µg in 0.5 µL per side) was infused over a one minute period. The injectors, which extended 0.5mm (dHC) or 1mm (BLA) from the cannulae, were left in place for a further one minute to allow for drug diffusion before being removed. The rats were returned to their home cages for ten minutes before undergoing behavioural testing.

4.2.3 Histology

After behavioural testing was finished, the animals were deeply anaesthetised and then underwent cardiac perfusion with 0.9% saline followed by 4% paraformaldehyde. The brains were kept in 4% paraformaldehyde before being sectioned at 200µm or 60µm and stained for the presence of acetylcholinesterase (Koelle and Friedenwald, 1949).

4.2.4 Behavioural Procedures

The behavioural procedures used in this chapter have been outlined in chapter two. Briefly, in the contextual fear experiments SCH 23390 or saline was infused into the dHC or BLA ten minutes prior to CFC (four 0.5mA, one second footshocks). The rats underwent two minute retention tests twenty four hours and seven days later. These were video recorded and the rats' freezing behaviour was subsequently scored. In the open field experiments, SCH 23390 or saline was infused into the dHC or BLA ten minutes prior to placement in the open field arena for ten minutes. The total distance moved and time spent in the inner-zone of the arena for each rat was obtained using Ethovision software. Rearing behaviour was assessed manually by the experimenter at the time of testing.

4.2.5 Data Analysis

Two-way mixed ANOVA was used to analyse the freezing levels, with drug (SCH 23390 or vehicle) as the between-subject factor and memory retention session (24 hr and 7 d) as the within-subject factor. Open field data were analysed using unpaired t-tests to compare the total distance moved, the rearing frequency and percentage of time spent in the centre of the open field between the drug and saline treated groups. All data are presented as the mean + SEM.

4.3 Results

4.3.1 Cannulae Placements in the dHC and BLA

Out of the sixty two rats used in these experiments nineteen were excluded due to illness after surgery (n=1), missed placements (n=10), or poor histology (due to using inappropriate equipment) leading to inability to see the cannulae tips (n=8). Schematic representations of the cannula placements in the dHC and BLA are presented in Fig 4.1.

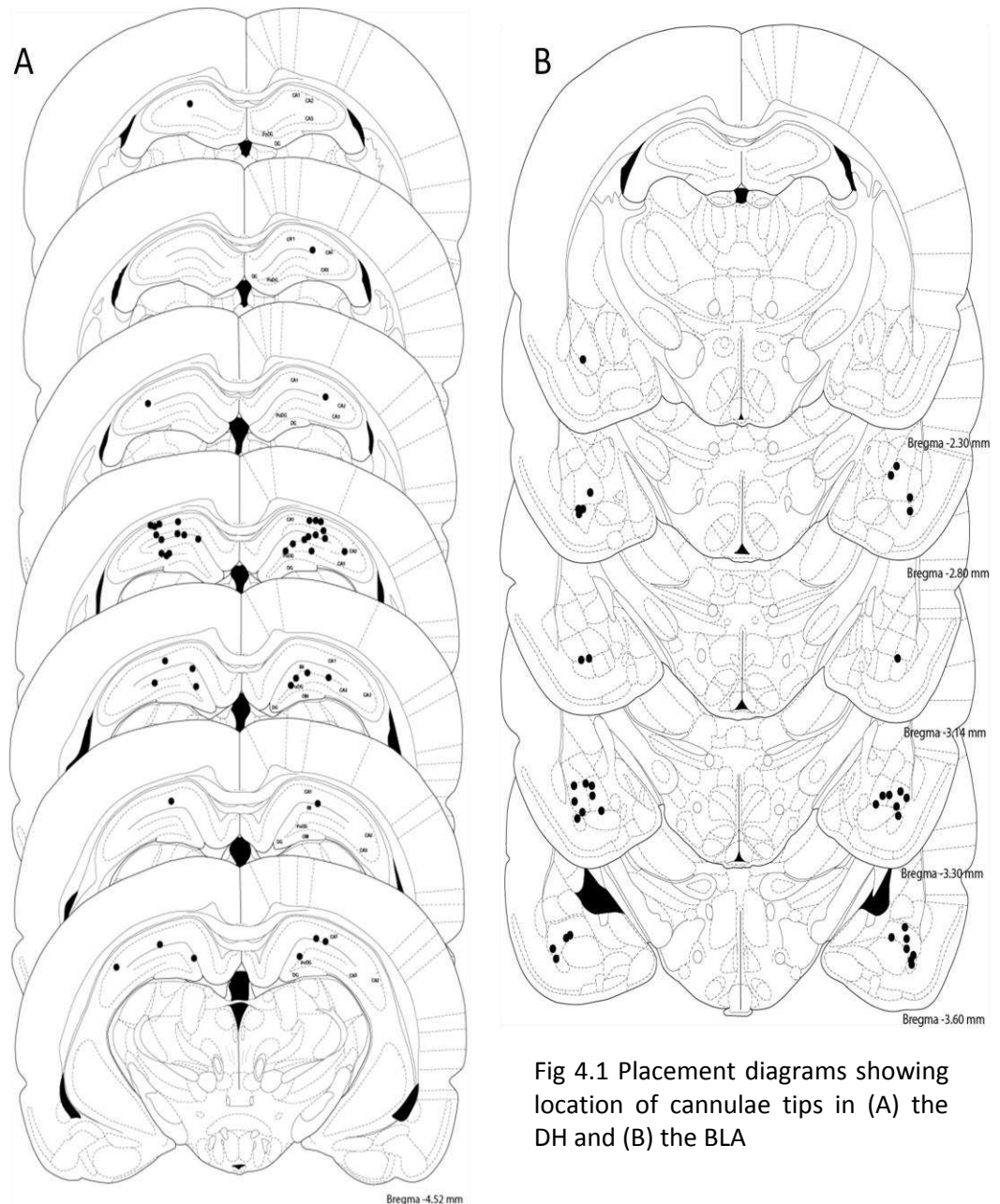


Fig 4.1 Placement diagrams showing location of cannulae tips in (A) the DH and (B) the BLA

4.3.2 Effect of Intra-dHC D1 Receptor Antagonism on Contextual Fear Acquisition

The effects of intra-dHC SCH 23390 or saline infusion ten minutes before conditioning are presented in Fig 4.2. Analysis of the intra-dHC experiment (SCH 23390 $n=12$, saline $n=11$) using a two-way mixed ANOVA revealed a main effect of drug ($F_{(1,21)}=5.153$, $P<0.05$) but no drug \times time interaction ($F_{(1,21)}=0.863$, $P>0.05$). As SCH 23390 was found to effect behaviour in the open field when administered into the dHC thirty minutes prior to testing (see

below), the data for freezing behaviour during the last two minutes of the conditioning session was not included in the analysis as non-specific effects of the drug could not be ruled out.

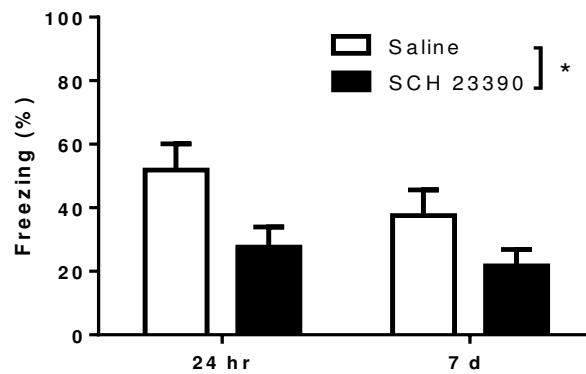


Fig 4.2 The effect of intra-DH D1R antagonism on contextual fear. SCH23390 prior to conditioning impaired memory during retention sessions (* $P < 0.05$).

4.3.3 Effect of Intra-BLA D1 Receptor Antagonism on Contextual Fear Acquisition

Analysis of intra-BLA infusion of SCH 23390 ($n=9$) ten minutes before conditioning compared with saline ($n=11$) revealed no significant main effect of drug ($F_{(1,17)}=0.964$, $P > 0.05$) and no drug x time interaction ($F_{(1,17)}=0.050$, $P > 0.05$) (Fig 4.3). As SCH 23390 was found to effect behaviour in the open field when administered into the BLA thirty minutes prior to testing (see below), the data for freezing behaviour during the last two minutes of the conditioning session was not included in the analysis as non-specific effects of the drug could not be ruled out. Baseline freezing behaviour was decreased in the animals with BLA cannulae. This could be due to partial BLA damage resulting from the implants.

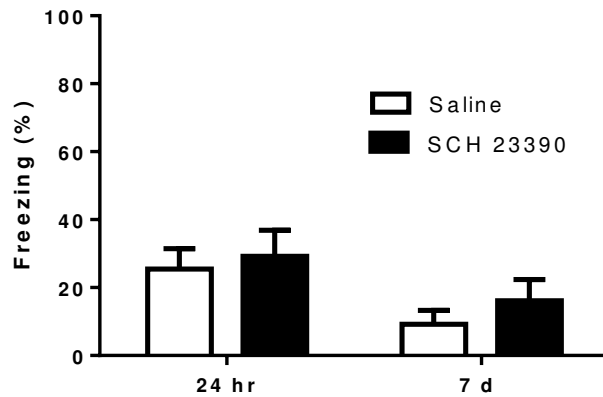


Fig 4.3 The effect of intra-BLA D1R antagonism on contextual fear. SCH23390 prior to conditioning did not affect freezing during memory retention sessions.

4.3.4 Effect of Intra-dHC D1 Receptor Antagonism on Behaviour in the Open Field

The effect of intra-dHC or intra-BLA SCH 23390 infusions on behaviour in the open field was tested (see Fig 4.4 and 4.5). For intra-dHC infusions, unpaired t-tests revealed that SCH 23390 (n=13) compared with saline (n=10) significantly decreased the total distance moved ($t_{(21)}=3.254$, $P<0.01$), rearing frequency ($t_{(21)}=4.595$, $P<0.01$) and time spent in the inner-zone ($t_{(16)}=5.371$, $P<0.01$).

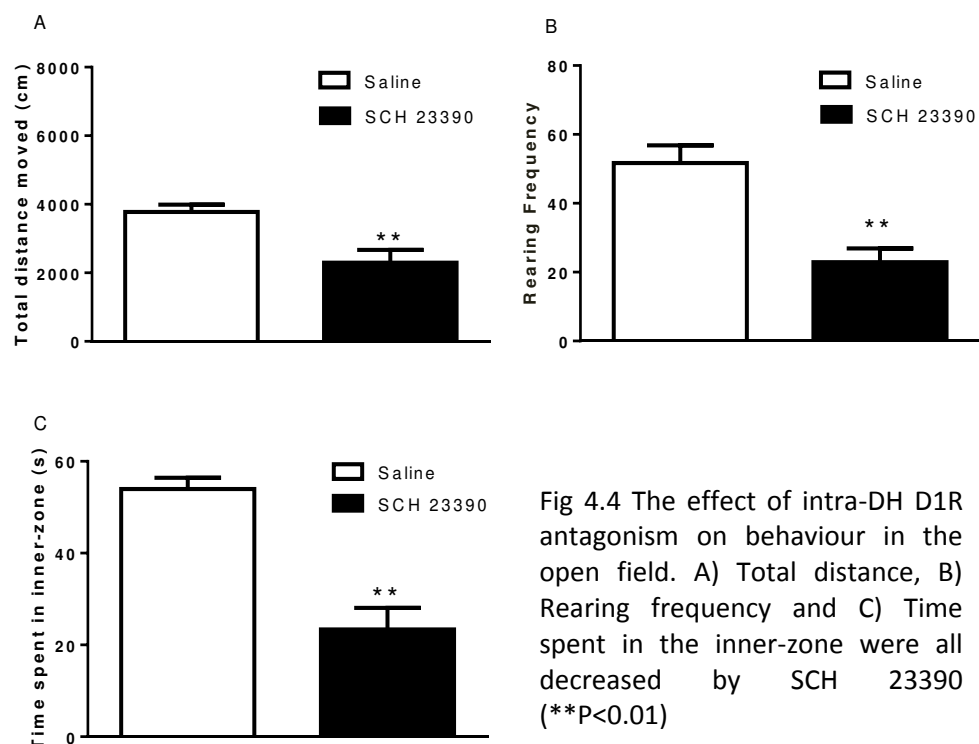


Fig 4.4 The effect of intra-DH D1R antagonism on behaviour in the open field. A) Total distance, B) Rearing frequency and C) Time spent in the inner-zone were all decreased by SCH 23390 (** $P<0.01$)

4.3.5 Effect of Intra-BLA D1 Receptor Antagonism on Behaviour in the Open Field

For the intra-BLA open field experiment total distance moved was found to be reduced in SCH 23390 group (n=8) compared with the saline group (n=10). Unpaired t-tests revealed a significant effect on total distance moved ($t_{(16)}=4.410$, $P<0.01$) but not rearing frequency ($t_{(16)}=1.735$, $P>0.05$) or time spent in the inner-zone ($t_{(16)}=0.163$, $P>0.05$).

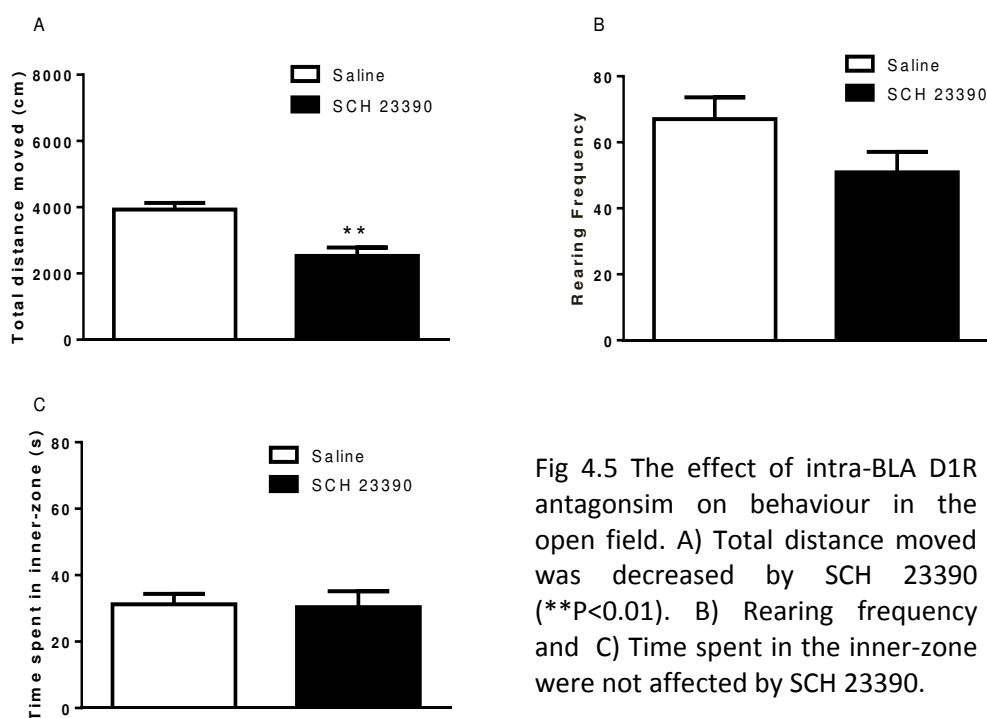


Fig 4.5 The effect of intra-BLA D1R antagonism on behaviour in the open field. A) Total distance moved was decreased by SCH 23390 (**P<0.01). B) Rearing frequency and C) Time spent in the inner-zone were not affected by SCH 23390.

4.4 Discussion

D1R antagonism in the dHC impaired contextual fear memory acquisition. Administration of SCH 23390 into the dHC prior to CFC resulted in reduced freezing behaviour during LTM retention testing twenty four hours and seven days later. Conversely, D1R antagonism in the BLA was not found to impair contextual fear memory acquisition. Intra-BLA SCH 23390 administered prior to CFC did not reduce freezing during LTM testing. Both intra-dHC and intra-

BLA infusions of SCH 23390 reduce total distance moved in the open field ten minutes after administration.

4.4.1 D1 Receptor Antagonism in the Hippocampus during Contextual Fear Acquisition

Intra-dHC SCH 23390 infusion prior to CFC was found to impair contextual fear acquisition. To the best of our knowledge this has not been investigated before. It has been proposed that D1Rs are involved in the synthesis of PRPs (Wang et al., 2010; Sajikumar and Frey, 2004). It could be that intra-dHC SCH 23390 prior to CFC impairs the initiation of PRP synthesis and so E-LTP is not being converted to late L-LTP, leading to impaired LTM. An alternative explanation could be that attention is required during acquisition for the initial association between CS and US or for contextual encoding itself to occur and D1R antagonism could be impairing this. It has been shown that attention increases stability of hippocampal place fields and that SCH 23390 interferes with this (Kentros et al., 2004). The finding that intra-dHC SCH 23390 impairs CFC acquisition is in agreement with previous research investigating spatial and episodic-like memory. Intra-dHC SCH 23390 fifteen minutes before a new location trial in the DMP watermaze was found to impair memory six hours later (O'Carroll et al., 2006). In a modification of the DMP water maze which included an additional more sensitive measure (search preference), SCH 23390 infusions before training were also found to impair memory thirty minutes later (Pezze and Bast, 2012). Episodic-like memory was also impaired by Intra-dHC SCH 23390 prior to training during retention tests twenty four hours but not thirty minutes later (Bethus et al., 2010). However, previous research investigating the effects of SCH 23390 in the hippocampus on other memory paradigms including object recognition and the radial arm maze, found that D1R antagonism did not impair initial memory formation, in disagreement with our findings. SCH 23390 infusions into the hippocampus before or after training did not impair object recognition memory (Balderas et al., 2013; Rossato et al., 2013). Intra-dHC

infusions of SCH 23390 prior to working memory in the radial-arm maze was not affected by SCH 23390 infusions into the vHC (Wilkerson and Levin, 1999).

4.4.2 D1 Receptor Antagonism in the Amygdala during Contextual Fear Acquisition

Intra-BLA infusion of SCH 23390 was not found to affect the acquisition of contextual fear memory. This is not in agreement with another study which found that SCH 23390 impaired the acquisition and retention of auditory and background contextual fear when infused into the amygdala (Guarraci et al., 1999). However, drug infusions were made into the cAMY of female Long Evans rats in that study, as opposed to the BLA of male Lister hooded rats in the present study; these variations could explain the different results obtained. Also, in the Guarraci et al. (1999) study contextual freezing behaviour was measured in response to the background context during the interval between tone presentations. Therefore it is difficult to say whether the rats were freezing due to cued associations induced by the tone or strictly to the contextual associations of the chamber. There are mixed findings on the effects of amygdala D1R antagonism in other memory paradigms. In one study, FPS acquisition was not impaired by intra-amygdala SCH 23390 administered prior to training (De Oliveira et al., 2011) whereas another study found that intra-amygdala SCH 23390 prior to FPS training did impair subsequent memory retention (Greba and Kokkinidis, 2000). The lack of effect of intra-BLA SCH 23390 prior to CFC could potentially be due to compensation by other brain regions. The dynamic memory theory postulates that the hippocampus and amygdala under normal circumstances are necessary for contextual fear acquisition; however, when damaged other competing brain regions can compensate (Fanselow, 2010). The bed nucleus of the stria terminalis has been shown to be able compensate for loss of the BLA (Poulos et al., 2010). Lesions of both the BLA and the bed nuclei of stria terminalis prevented acquisition of contextual fear after extensive over training whereas BLA lesions alone did not (Poulos et al., 2010). In another study, infusions of SKF 38393 (D1R agonist) concurrently into both the

amygdala and mPFC were required to overcome the object recognition memory impairment produced by VTA inactivation (Rossato et al., 2013).

4.4.3 D1 Receptor Antagonism in the Hippocampus and Amygdala on Behaviour in the Open Field

Intra-dHC or intra-BLA SCH 23390 infusions ten minutes prior to open field testing resulted in a reduction in total distance moved. Intra-dHC SCH 23390 also led to reduced rearing frequency and time spent in the inner zone of the open field, whereas intra-BLA SCH 23390 did not affect these behaviours. These results are in conflict with previous research. Liao et al. (2013) found no effect of intra-CA1 SCH 23390 on locomotion in the open field, but a lower dose (30 ng) was used in their study as opposed to the 5µg per side used in our experiment (Liao et al., 2013). SCH 23390 was also not found to reduce locomotion and rearing behaviour when infused into the vHC (Gimenez-Llort et al., 2002) but again a lower dose of SCH 23390 was used and drug infusions were made into the ventral as opposed to the dHC. Other studies have also shown no effects of SCH 23390 on locomotion in the open field when infused into BLA or the IPCs before testing (de Oliveira et al., 2011; De la Mora et al., 2005). Again, lower doses of SCH 23390 were used in these studies which could explain why the results differ from ours. The time spent in the inner-zone of the open field is hard to interpret given that both intra-dHC and intra-BLA SCH 23390 infusions were found to reduce total distance moved; therefore the reduction seen with intra-dHC SCH 23390 is most likely a result of decreased locomotion rather than drug induced increases in anxiety.

4.4.4 Chapter Four Summary

The effect of D1R antagonism in the dHC and BLA on contextual fear acquisition was investigated. It was found that SCH 23390 infusions into the dHC but not the BLA impaired freezing behaviour during LTM retention sessions. D1R activation during contextual fear acquisition in the dHC could be required for the initial synthesis of PRP (Wang et al., 2010; Sajikumar and Frey, 2004). It is likely that D1Rs in the dHC are important for the encoding of

spatial representations of the fear context. It is also possible that the dHC has a role in the association of the context with the US. The experiments outlined in this chapter have not previously been undertaken for contextual fear memory and therefore extend the existing literature.

Chapter Five

**Systemic D1 Receptor Antagonism during
Contextual Fear Retrieval,
Reconsolidation and Extinction**

5.1 Introduction

The cAMP/PKA and PLC molecular pathways which are regulated by D1Rs both lead to the activation of the transcription factor CREB. CREB activation initiates the synthesis of new proteins that can be used for plasticity related changes such as synthesis of structural proteins or ion channel modulation. Previous research has shown CREB to be involved in the processes of retrieval, reconsolidation and extinction. In one study increases in CREB occurred in the hippocampus and amygdala following contextual and auditory fear reactivation (Kida et al., 2002; Mamiya et al., 2009). However, another study found increased pCREB in the amygdala but not the hippocampus following cued fear retrieval (Hall et al., 2001). Activation of neurons which had high CREB activity at the time of conditioning was found to be sufficient to retrieve an auditory fear memory without behavioural cues (Kim et al., 2014) and ablation of these specific neurons impaired retrieval (Han et al., 2009). Transgenic mice in which CREB was repressed in the hippocampus and amygdala were found to have impaired contextual and auditory fear reconsolidation (Kida et al., 2002). CREB repressed mice were found to have impaired contextual fear extinction (Mamiya et al., 2009) and pCREB was increased in the mPFC and amygdala but not hippocampus following extinction training (Mamiya et al., 2009).

There are mixed findings for the role of D1Rs in memory retrieval. In one such study systemic SKF 81297 (D1R agonist) enhanced the retrieval of object recognition at a delay of four hours whereas at a delay of fifteen minutes the opposite was found, with the drug impairing retrieval (Hotte et al., 2005). SCH 23390 infused into the nucleus accumbens shell lead to enhanced retrieval of contextual fear (Albrechet-Souza et al., 2013). SCH 23390 infusions into either the VTA or the amygdala on the other hand, had no effect on the retrieval of FPS (De Oliveira et al., 2009; Greba and Kokkinidis, 2000). Intra-hippocampal SCH 23390 was not found to impair the retrieval of inhibitory avoidance in one study (Izquierdo et al., 1998), whereas another

study found that it did, but that intra-amygdala SCH 23390 did not (Barros et al., 2001). Other research showed the involvement of D1Rs in the retrieval of fear using second order auditory fear conditioning (Nader and LeDoux, 1999). It has previously been shown that systemic D1R antagonism is not required for the retrieval of contextual fear (Inoue et al., 2000).

The role of systemic D1Rs in the reconsolidation or extinction of contextual fear has not, to the best of our knowledge, been investigated before. However, studies testing other memory paradigms using D1R agonists and antagonists have produced mixed results, some of which have been outlined below. Systemic amphetamine administration immediately following a reactivation session was not found to affect reconsolidation of PCA (Blais and Janak, 2007) but did enhance the reconsolidation of CPP (Blais and Janak, 2006). Systemic SCH 23390 was found to impair the reconsolidation of passive avoidance in chicks (Sherry et al., 2005) and SCH 23390 infused into the mPFC of non-habituated, but not habituated, rats resulted in impaired object recognition reconsolidation (Maroun and Akirav, 2009). In one study investigating the involvement of D1Rs in extinction using transgenic mice, D1R KO mice did not have impaired contextual or auditory fear extinction (Ortiz et al., 2010), whereas another found that extinction of contextual fear and passive avoidance was impaired (EL-Ghundi et al., 2001). Systemic administration of MPD before or after contextual fear was found to improve extinction (Abraham et al., 2012). Other studies found no effect of systemic amphetamine on auditory fear extinction (Mueller et al., 2009; Carmack et al., 2010). Auditory fear extinction was found to be impaired by intra-PFC but not intra-BLA infusion of SCH 23390 (Hikind and Maroun, 2008). But another study found that intra-PFC and BLA infusions of SCH 23390 did not affect the extinction of contextual fear or inhibitory avoidance, whereas intra-hippocampal infusions did (Fiorenza et al., 2012). Systemic SCH 23390 was found to impair the extinction of CPP (Fricks-Gleason et al., 2012) and D1R agonists, including SKF 38393, blocked FPS extinction (Borowski and Kokkinidis, 1998)

Chapter Five Aims

The experiments in this chapter extend previous research by investigating the effects of systemic D1R antagonism on the reconsolidation and extinction of contextual fear in rats. The aim was to test the effect of administration of SCH 23390 prior to or immediately after either a short memory reactivation session or a longer extinction session.

5.2 Methods

The methods used in this chapter have been outlined in chapter two. Briefly, in the reconsolidation experiments SCH 23390 or saline was injected thirty minutes before (SCH 23390 n=10; saline n=10) or immediately after (SCH 23390: n=9; saline n=8) a reactivation session in which the rats were returned to the conditioning chamber for two minutes in the absence of footshocks. The rats had their memory tested twenty four hours and seven days later by again returning them to the chamber for two minutes. As SCH 23390 or saline was injected before reactivation in some rats, this enabled memory retrieval to also be tested. For the extinction experiments SCH 23390 or saline was injected thirty minutes before (SCH 23390: n=9; saline: n=9) or immediately after (SCH 23390: n=10; saline: n=9) a twenty minute extinction session in which the rats were returned to the conditioning chamber in the absence of footshocks.

For the reconsolidation experiment, a two-way mixed ANOVA was used to analyse the freezing levels, with drug (SCH 23390 or vehicle) as the between-subject factor and memory testing session (REACT, PR-LTM1 and PR-LTM7) as the within-subject factor. For the extinction experiment in which SCH 23390 or saline was administered prior to the extinction session, drug effects on extinction and its retention were examined separately. Two separate two-way ANOVAs were used, with drug (SCH 23390 or vehicle) as the between-subject factor and testing session (early or late extinction) as the within-subject factor in the first analysis, and extinction retention (24 hr and 7 d) as the within-subject factor in the second analysis. For the extinction experiment in which

SCH 23390 or saline was administered immediately after the extinction session, a two-way ANOVA was used with the within-subject factor being extinction retention (24 hr and 7 d). All data are presented as the mean + SEM.

5.3 Results

5.3.1 Effect of Systemic D1R Antagonism on Contextual Fear Retrieval and Reconsolidation

Administration of SCH 23390 thirty minutes prior to contextual fear reactivation did not impair freezing behaviour during reactivation or PR-LTM testing twenty four hours or seven days later (see Fig5.1). Analysis using a two-way ANOVA revealed no main effect of drug ($F_{(1,18)}=0.888$, $P>0.05$) and no drug x time interaction ($F_{(3,54)}=1.780$, $P>0.05$). Likewise, SCH 23390 administered immediately after reactivation had no effect on freezing twenty four hours or seven days later, as there was no main effect of drug ($F_{(1,15)}=0.545$, $P>0.05$) and no interaction between drug and time ($F_{(3,45)}=1.536$, $P>0.05$).

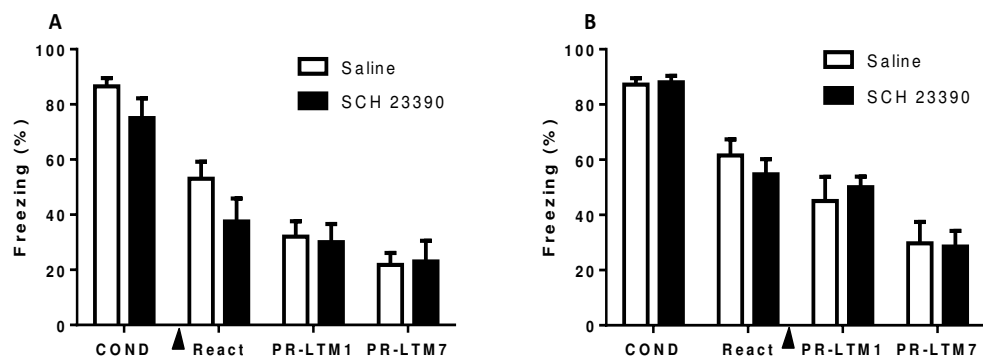


Fig 5.1 The effect of D1R antagonism on contextual fear reconsolidation. SCH 23390 A) prior to or B) immediately after reactivation (REACT) did not impair freezing 24hrs (PR-LTM1) or 7days (PR-LTM7) later. Conditioning (COND); arrow denotes time of drug administration.

5.3.2 Effect of Systemic D1R Antagonism on Contextual Fear Extinction

There was also no difference between freezing behaviour in the drug and saline groups during early and late extinction with no main effect of drug ($F_{(1,16)}=0.817$, $P>0.05$) and no drug x time interaction ($F_{(1,16)}=0.241$, $P>0.05$). Administration of SCH 23390 thirty minutes prior to contextual fear extinction did not impair freezing behaviour twenty four hours or seven days later (see Fig5.2). Analysis using a two-way ANOVA revealed no main effect of drug ($F_{(1,16)}=0.139$, $P>0.05$) and no drug x time interaction ($F_{(1,16)}=0.570$, $P>0.05$). Likewise SCH 23390 administered immediately after the extinction session had no effect on freezing twenty four hours or seven days later, as there was no main effect of drug ($F_{(1,17)}=1.079$, $P>0.05$) and no drug x time interaction ($F_{(1,17)}=0.049$, $P>0.05$).

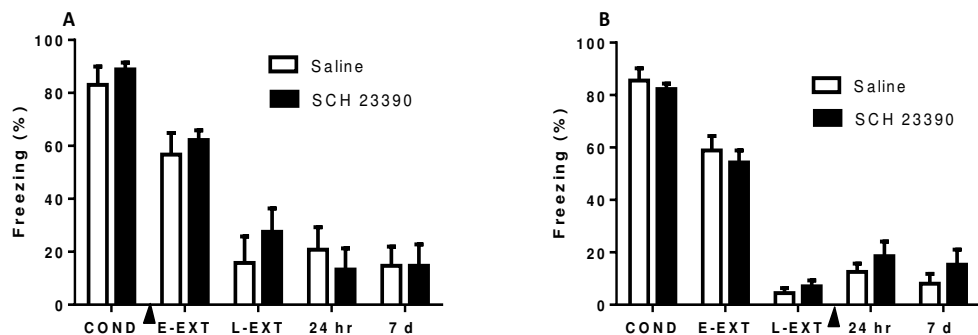


Fig 5.2 The effect of D1R antagonism on contextual fear extinction. A) SCH 23390 prior to or B) immediately after extinction (EXT) had no effect on memory retention 24hrs and 7days later. Conditioning (COND), early extinction (E-EXT), late extinction (L-EXT), arrow denotes time of drug administration.

5.4 Discussion

Systemic D1R antagonism was not found to effect contextual fear memory retrieval, reconsolidation, or extinction. This indicates that DA acting at the D1R is not required during these memory stages.

5.4.1 Systemic D1 Receptor Antagonism during Retrieval

Systemic administration of SCH 23390 was not found to affect the retrieval of contextual fear. This is in agreement with previous research for contextual

fear (Inoue et al., 2000). This is also in agreement with previous research looking at FPS. SCH 23390 infused into either the VTA (De Oliveira et al., 2009) or the amygdala (Greba and Kokkinidis, 2000) was not found to effect FPS retrieval. Intra-hippocampal SCH 23390 was not found to affect the retrieval of inhibitory avoidance, in agreement with the present results (Izquierdo et al., 1998). However in another study intra-hippocampal SCH 23390 impaired retrieval of inhibitory avoidance, whereas intra-amygdala SCH 23390 did not (Barros et al., 2001). Other research is also not in agreement, SCH 23390 infused into the nucleus accumbens shell enhanced the retrieval of contextual fear (Albrechet-Souza et al., 2013) and intra-BLA SCH 23390 impaired the retrieval of cued fear (Nader and LeDoux, 1999).

5.4.2 Systemic D1 Receptor Antagonism during Reconsolidation

Systemic SCH 23390 administered prior to or immediately after reactivation was not found to affect contextual fear reconsolidation. This has not been shown before to the best of our knowledge. These findings are not in agreement with previous systemic research investigating the reconsolidation of passive avoidance in chicks (Sherry et al., 2005). SCH 23390 infused into the mPFC was also found to impair the reconsolidation of object recognition in non-habituated rats (Maroun and Akirav, 2009). However intra-mPFC SCH 23390 infusions did not affect the reconsolidation of object recognition in habituated rats (Maroun and Akirav, 2009), in agreement with the present findings.

5.4.3 Systemic D1 Receptor Antagonism during Extinction

Systemic administration of SCH 23390 prior to or immediately after extinction was not found to affect extinction acquisition or subsequent extinction memory retention twenty four hours and seven days later. These findings are not in agreement with previous systemic research where SCH 23390 was found to impair the extinction of CPP (Fricks-Gleason et al., 2012). Other studies have found that intra-PFC SCH 23390 impairs the extinction of auditory fear (Hikind and Maroun, 2008), but not inhibitory avoidance

(Fiorenza et al., 2012). Intra-BLA SCH 23390 was not found to affect the extinction of auditory fear (Hikind and Maroun, 2008) or inhibitory avoidance, whereas intra-hippocampal infusions did (Fiorenza et al., 2012).

It is possible that the dose of SCH 23390 used in the experiments carried out in this chapter was not strong enough to produce an effect. However, the same dose was found to impair the acquisition of contextual fear (Chapter 3) and also reduced locomotion in the open field (Chapter 3) indicating that this is most likely not the case. It is also possible that SCH 23390 is having opposite effects in different brain regions and therefore no net effect is seen when the drug is administered systemically. This could be resolved by future studies in which SCH 23390 is administered centrally into different brain regions such as the hippocampus and the effect on contextual fear retrieval, reconsolidation and extinction determined.

5.4.4 Chapter Five Summary

It was found that D1R antagonism is not involved in the retrieval, reconsolidation and extinction of contextual fear. This could be because DA is not involved or it is possible that the dose of SCH 23390 used in the experiments was not high enough to affect the modulation of these processes. It is also possible that D2Rs are important instead. Previous research has implicated D2Rs in the retrieval of FPS (De Oliveira et al., 2006), the reconsolidation of CPP (Yan et al., 2013) and the extinction of auditory fear (Mueller et al., 2010; Holtzman-Assif, 2010; Ponnusamy et al., 2005). Future studies could investigate the role of D2Rs in contextual fear retrieval, reconsolidation and extinction.

Chapter Six

**Systemic D1 Receptor Antagonism during
Contextual Fear Memory Destabilization**

6.1 Introduction

Memory destabilization occurs when a previously consolidated memory is retrieved and becomes unstable. It has been proposed that prediction error, which occurs with a discrepancy between expected and actual events, is required to engage the process of destabilization (Ben Mamou et al., 2006). Once a memory has been destabilized it must undergo the process of reconsolidation in order to be stabilized again. It was recently demonstrated that FPS memory reconsolidation in humans only occurred where a positive or negative prediction error was present during the reactivation session (Sevenster et al., 2013). Normally strong memories are less likely to undergo reconsolidation. However the presence of a temporal prediction error resulted in destabilization and subsequent impairment of reconsolidation even for strong memories (Diaz-Mataix et al., 2013). The VTA was also shown to be important for Pavlovian over-expectation learning following a negative prediction error (Takahashi et al., 2009).

The first study to investigate the destabilization process showed that NMDARs are important in the process (Ben Mamou et al., 2006). They developed a method in which it was possible to show that the process of destabilization had been engaged by using the drug anisomycin. Anisomycin is a protein synthesis inhibitor which has been shown to impair PR-LTM when administered immediately following a reactivation session (Nader et al., 2000). They therefore argued that if the NMDAR antagonist ifenprodil administered before the reactivation session was able to prevent the memory impairing effect of administration of anisomycin immediately after reactivation, then the destabilization must have been blocked by ifenprodil, indicating the requirement for NMDARs in the process (Ben Mamou et al., 2006). Using this method but replacing anisomycin with MK-801 it was recently shown that VTA activity is involved in the destabilization of appetitive goal-tracking memory (Reichelt et al., 2013). Due to this building evidence demonstrating an involvement of prediction error in the destabilization process and evidence showing DA is required for prediction error (Schultz,

2013), we hypothesised that D1Rs could be important in the destabilization of contextual fear memories.

Chapter Six Aims

The aim of the study undertaken in this chapter was to investigate the effect of systemic SCH 23390 administration before and MK-801 immediately after a reactivation session, to test if impairing D1R activity would protect the memory from subsequent disruption by MK-801 and therefore indicate a role for D1Rs in contextual fear memory destabilization. For this to be relevant it must also be shown that MK-801 administered immediately after reactivation impaired reconsolidation.

6.2 Methods

The methods used in this chapter have been outlined in chapter two. Briefly, rats underwent CFC and were injected systemically twenty four hours later, with the SCH 23390/MK-801 group (n=9) receiving SCH 23390 thirty minutes before and MK-801 immediately after a reactivation session in which they were returned to the conditioning chamber for two minutes. The saline/MK-801 group (n=8) received saline before and MK-801 after the reactivation session and the saline/saline group (n=10) received saline both before and after. The rats had their PR-LTM tested twenty four hours and seven days later by again returning them to the chamber for two minutes. A two-way mixed ANOVA was used to analyse the freezing levels, with drug group (SCH 23390/MK-801, saline/MK-801 or saline/saline) as the between-subject factor and memory testing session (REACT, PR-LTM1 or PR-LTM7) as the within-subject factor. All data are presented as the mean + SEM.

6.3 Results

There were no differences between any of the groups during retrieval or PR-LTM (see Fig6.1). Analysis using a two-way ANOVA revealed no main effect of drug ($F_{(1,24)}=0.316$, $P>0.05$) and no drug x time interaction ($F_{(6,72)}=0.302$, $P>0.05$).

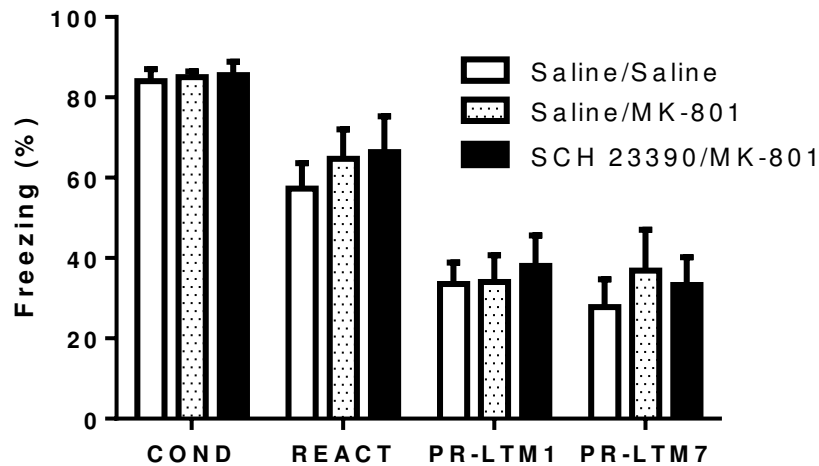


Fig 6.1 The effect of D1R antagonism on contextual fear memory destabilization. There was no difference in freezing behaviour between the SCH 23390/MK-801, saline/saline and saline/MK 801 groups 24hrs (PR-LTM1) or 7 days (PR-LTM7) later. Conditioning (COND), reactivation (REACT).

6.4 Discussion

It was found that SCH 23390 prior to reactivation and MK-801 immediately after had no effect on PR-LTM twenty four hours and seven days later. However, because administering saline before and MK-801 immediately after reactivation also had no effect, it is not possible to draw any conclusions on the role of D1R antagonism in contextual fear destabilization. The study depended on the Saline/MK-801 group having impaired PR-LTM but this was not found. Previous work has shown that systemic MK-801 administered after reactivation impaired contextual fear reconsolidation in mice (Charlier and Tirelli, 2011). It has also previously been shown that systemic MK-801 administered after reactivation impaired appetitive goal tracking reconsolidation in rats (Reichelt et al., 2013). These studies are not in agreement with our findings. However, in another study systemic MK-801 prior to reactivation of appetitive memory did not affect reconsolidation, in agreement with our findings (Lee and Everitt, 2008).

The lack of effect of MK-801 on contextual fear could be due to the reactivation session being too long in duration, leading to extinction rather than reconsolidation being engaged, or too short in duration, leading to destabilization only being partially induced (Bustos et al., 2009). It could also be that the 0.1mg/kg dose of MK-801 used was not sufficient to effect the reconsolidation of contextual fear. However, in our initial validation study (Chapter 2) we found that a 0.1mg/kg dose of MK-801 administered before a two minute reactivation did block reconsolidation, which would indicate this is most likely not the case. However, future studies could explore further the parameters under which MK-801 given after reactivation might disrupt reconsolidation. A weaker conditioning procedure could be used, the length of the reactivation session could be altered, and/or a higher dose of MK-801 could be administered. These procedural changes might potentially result in blockage of reconsolidation by MK-801 administration immediately after reactivation. Alternatively, future studies could replace MK-801 with a drug that impairs contextual fear reconsolidation when administered after reactivation to determine whether or not D1Rs are involved in contextual fear destabilization. Possible drug options are the GluN2A preferring NMDAR antagonist NVP-AAM077, the noradrenergic β -blocker propranolol or midazolam which enhances the effects of GABA at the GABA_A receptor (Milton et al., 2013; Debiec and LeDoux, 2004; Bustos et al., 2009).

Chapter Seven

General Discussion

7.1 Summary

The role of D1Rs in contextual fear learning and memory was investigated. The experiments outlined in chapters three and four found that systemic and intra-DH administration of SCH 23390 impaired contextual fear memory acquisition whereas intra-BLA SCH 23390 did not. The effect of intra-dHC and intra-BLA D1R antagonism during CFC acquisition has not been investigated before, therefore these findings advance the current literature.

The experiments outlined in chapter five found that systemic administration of SCH 23390 was not involved in the reconsolidation, retrieval or extinction of contextual fear memory. The effect of systemic D1R antagonism during CFC reconsolidation and extinction has not been investigated before and advances the understanding of the role of DA in these processes.

The experiments outlined in chapters two and six found that systemic administration of MK-801 impaired contextual fear reconsolidation when administered prior to, but not immediately after, memory reactivation. This, to the best of our knowledge, has not previously been demonstrated in rats and therefore extends the literature on the role of NMDARs in reconsolidation.

The experiment outlined in chapter six, looking at whether D1Rs are involved in contextual fear memory destabilization, was inconclusive. Future studies that could resolve these have therefore been outlined below, along with a discussion of the all the findings presented in this thesis.

7.2 MK-801 Effects on Acquisition and Reconsolidation

The finding that systemic MK-801 administration prior to CFC impairs subsequent memory retention is in agreement with previous systemic research (Gould et al., 2002). The finding that systemic MK-801 administration prior to CFC reactivation impairs reconsolidation has not, to the best of our knowledge, been tested before. This finding is in agreement

with previous research for auditory fear (Lee et al., 2006), appetitive memory (Lee and Everitt, 2008) and associative drug-seeking memory (Milton et al., 2008). There are, however, a number of alternative explanations for the above findings which are worth discussing. Firstly, it could be that reduction in freezing behaviour in the MK-801 injected rats during the reactivation sessions are a result of non-specific side effects on locomotion. Previous studies have found that MK-801 increases locomotion in the open field and Morris water maze at a dose of 0.1mg/kg (Wegener et al., 2011). Secondly, it is possible that the effects of MK-801 on memory retention twenty four hours after conditioning or reactivation could be due to state-dependency, which has previously been shown to occur for passive avoidance memory using MK-801 (Flint et al., 2013). A future study could test this by administering MK-801 prior to LTM or PR-LTM, testing to see if memory is still impaired by MK-801 administration prior to conditioning or reactivation, respectively.

Thirdly, a bigger difference between the freezing behaviour of MK-801 and saline groups at twenty four hours, compared with seven days after conditioning and reactivation, was found in both experiments and is a trend seen throughout the experiments presented in this thesis. This could be because extinction occurred due to the rats being exposed to the chamber without footshock, resulting in the rats forming a new competing non-fearful memory of the chamber. It could also be because the saline injected rats forgot their conditioning experience due to the passing of time. To test whether the rats had forgotten due to the passing of time, an experiment could be carried out in which the rats only have their memory tested seven days after conditioning (i.e. instead of both 24 hr and 7 d after conditioning). If the rats had a similar level of freezing to the rats in our experiment, then this would indicate that the rats forget the conditioning experience after a week. To test whether the rats had undergone extinction, one group of rats could be fear conditioned and then returned to the chamber for two minute sessions without footshock, on two occasions within a week (similar to the reconsolidation experiment), and another group could undergo one return

session (similar to the consolidation experiment). If the rats had similar levels of freezing to the rats in our experiment, then this would indicate that extinction had occurred.

The finding that systemic MK-801 administration immediately after CFC reactivation does not impair reconsolidation in rats has not previously been tested and is in agreement with work carried out on appetitive memory (Lee and Everitt, 2008). However, other research has found that MK-801 immediately after CFC reactivation did impair reconsolidation in mice (Charlier and Tirelli, 2011) and appetitive goal-tracking memory in rats (Reichelt et al., 2013). It is possible that these differences could be because of the experimental parameters used. It could be that reconsolidation is not being engaged in our experiment because the footshock protocol used during conditioning was too strong. Strong memories have been shown to be less likely to undergo reconsolidation than weaker ones (Suzuki et al., 2004). However, we found that MK-801 administered prior to reactivation impaired reconsolidation under the current footshock protocol (Chapter 2). It could be that the two minute reactivation session used in our experiments was not engaging the reconsolidation process. It has previously been shown that if the memory reactivation session is too short then contextual fear reconsolidation does not take place (Bustos et al., 2009). However, we found that MK-801 impaired reconsolidation using a two minute reactivation session when it was given before reactivation (Chapter 2). In conclusion, NMDARs are important for the initial acquisition of CFC and reconsolidation of CFC when MK-801 is administered systemically prior to, but not immediately after, reactivation.

7.3 Systemic and Central SCH 23390 Effects on Acquisition

Systemic D1R antagonism was found to modulate contextual fear memory acquisition. Administration of SCH 23390 prior to CFC, led to reduced freezing behaviour during subsequent retention tests twenty four hours later. This finding is in agreement with previous research (Inoue et al., 2000, Calzavara et al., 2009) and is most likely not due to side effects of SCH 23390 on

locomotion or pain sensitivity. SCH 23390 was not found to effect behaviour in the open field when administered twenty four hours prior to testing. SCH 23390 was also not found to affect the pain sensitivity threshold of the rats compared with saline injected controls. It is possible that D1R activation during contextual fear acquisition could be involved in the synthesis of PRPs. These proteins, such as kinase M ζ (Navakkode et al., 2010), have been proposed to be important for the conversion of E-LTP to L-LTP as describe by the synaptic tagging and capture hypothesis (Frey and Morris, 1997). A bigger difference between the controls and drug group at twenty four hours compared with seven days was observed. This is similar to what was found when MK-801 was administered prior to conditioning (Chapter 2). This could be due to the saline injected controls simply forgetting with the passing of time or it could be due to extinction as a result of the rats being exposed to the chamber without footshocks. Future experiments that could address this have been outlined above.

Infusion of SCH 23390 into dHC prior to CFC was found to impair freezing behaviour during LTM retention. This indicates that DA modulation in the dHC is important during the acquisition of contextual fear. This is in agreement with previous research investigating spatial and episodic-like memory (O'Carroll et al., 2006; Pezze and Bast, 2012; Bethus et al., 2010). It is possible that D1R activation in the dHC during contextual fear acquisition could lead to the synthesis of PRPs, such as PKM ζ , which are involved in the conversion of E-LTP to L-LTP as describe by the synaptic tagging and capture hypothesis (Frey and Morris, 1997). Similar to the findings for systemic SCH 23390 prior to CFC there was a bigger difference between the control and drug groups at twenty four hours compared with seven days when SCH 23390 was administered centrally. Future experiments that could address this have been outlined above.

Infusion of SCH 23390 directly into the BLA prior to CFC was not found to impair acquisition, thus indicating that DA modulation in this brain region is

not involved in CFC. This conflicts with previous research undertaken on auditory fear (Guarraci et al., 1999) and CFC (Biedenkapp and Rudy, 2009). There are mixed findings for the involvement of BLA DA modulation in the acquisition of FPS. One study found that FPS was impaired (Greba and Kokkinidis, 2000) whereas another study found that it was not, in agreement with our findings for contextual fear (De Oliveira et al., 2011). The lack of effect of intra-BLA SCH 23390 to modulate contextual fear acquisition could also be because other brain regions such as the BNST and IL are compensating (Fanselow, 2010). These brain regions receive DA innervations and express D1Rs (Swanson, 1982; Gustafson and Greengard, 1990; Lu et al., 1997) and are involved in contextual fear acquisition (Poulos et al., 2010; Zelikowsky et al., 2013).

In conclusion, SCH 23390 was found to impair contextual fear acquisition when administered systemically and centrally into the dHC but not when administered into the BLA. Understanding the role of contextual fear acquisition potentially provides insight into the mechanisms involved in the development of anxiety disorders such as PTSD. For example it could be that individual differences in D1R signalling in the dHC could determine why some people are more susceptible to the development of PTSD compared with others.

7.4 Systemic SCH 23390 Effects on Consolidation, Retrieval, Reconsolidation and Extinction

Systemic D1R antagonism was not found to modulate contextual fear consolidation or retrieval. Systemic administration of SCH 23390 immediately after CFC had no effect on freezing behaviour during retention tests twenty four hours or seven days later. Retrieval of contextual fear memory was not found to be affected by SCH 23390 administered prior to reactivation. These results are in agreement with previous literature (Inoue et al., 2000). It was also found that SCH 23390 administered either prior to, or immediately after, a short two minute reactivation session or longer twenty minute extinction

session, did not impair freezing behaviour during the reactivation session or during subsequent retention tests. This indicates that D1Rs are not involved in contextual fear memory reconsolidation or extinction, which has not previously been shown.

It could be that D2Rs rather than D1Rs are important for these contextual fear memory stages. Previous studies using D2Rs agonists and antagonists have indicated that D2R are important in other types of memory paradigms, some of which have been outlined below. Intra-BLA infusion of the D2R antagonist sulpiride was found to impair the consolidation of inhibitory avoidance (Lalumiere et al., 2004). Other research has shown the involvement of D2Rs in FPS retrieval. Systemic administration of the D2R agonist quinpirole impaired FPS retrieval (De Oliveira et al., 2006). Intra-VTA infusions of quinpirole were also found to impair FPS (Borowski and Kokkinidis 1996; De Oliveira et al., 2009). It has been suggested that quinpirole could be affecting pre-synaptic auto receptors resulting in decreased DA levels at VTA DA neuronal terminal fields (De Oliveira et al., 2009). One such terminal field is the BLA of the amygdala. Intra-VTA quinpirole was found to decrease DA levels in the BLA (De Oliveira et al., 2011). Intra-BLA infusion of the D2R antagonist sulpiride impaired FPS (De Oliveira et al., 2011). D2Rs have also been implicated in the reconsolidation of CPP to cocaine. Systemic administration of PG01037, a selective antagonist of the D₃ receptor, was found to impair reconsolidation of CPP when administered following memory reactivation (Yan et al., 2013). Systemic or intra-nucleus accumbens administration of haloperidol was found to impair auditory fear extinction (Holtzman-Assif et al., 2010). Systemic or intra-infralimbic administration of the D_{2/3} receptor antagonist raclopride impaired auditory fear extinction (Mueller et al., 2010). Another study found that auditory fear extinction in mice was enhanced by sulpiride (D2R antagonist) and partially impaired by quinpirole (D2R agonist) (Ponnusamy et al., 2005).

In conclusion D1Rs were not found to be involved in the consolidation, retrieval, reconsolidation or extinction of contextual fear. Understanding the involvement of D1Rs in reconsolidation and extinction is particularly important, given the potential of these memory stages in the treatment of anxiety disorders. The work in this thesis shows that D1Rs do not modulate these contextual fear processes, thus providing useful information for future hypothesis generation.

7.5 Systemic SCH 23390 Effects on Destabilization

The experiments carried out to determine whether D1R antagonism is involved in contextual fear destabilization were inconclusive due to the finding that MK-801 did not impair reconsolidation when administered immediately after contextual fear reactivation. In this study MK-801 was being used as a 'tool' in order to tease apart the role of D1Rs in destabilization. Conclusions therefore cannot be drawn regarding the lack of effect observed when SCH 23390 was administered prior to and MK-801 immediately after contextual fear reactivation. Future studies could replace MK-801 with an alternative 'tool' drug that does impair contextual fear when administered immediately after reactivation. Potential options have been discussed below.

One possibility would be a protein synthesis inhibitor such as anisomycin; however, systemic application of this drug has been reported to cause sickness in rats and is therefore probably best avoided. It has been demonstrated that GluN2A containing NMDARs are engaged in the reconsolidation process, whereas GluN2B containing NMDARs are involved in destabilization (Milton et al., 2013). Therefore rather than using MK-801, which is a non-selective NMDAR antagonist binding to both subunits, it might be sensible to use a GluN2A preferring NMDAR antagonist such as NVP-AAM077. This drug has previously been shown to impair the reconsolidation of auditory fear when administered into the BLA prior to reactivation (Milton et al., 2013) and other studies have used this drug systemically in rats and

mice (Chaperon et al., 2003). Alternative options are the noradrenergic β -blocker propranolol or the short-acting benzodiazepine midazolam, which enhances the activity of GABA at the GABA_A receptor. Systemic and intra-amygdala administration of propranolol has previously been shown to impair the reconsolidation of auditory fear when administered after reactivation in rats (Debiec and LeDoux, 2004). Likewise, systemic administration of midazolam immediately after contextual fear reactivation blocked reconsolidation in rats (Bustos et al., 2009).

7.6 Conclusion

In conclusion, the main findings of this thesis are that systemic and intra-dHC D1R antagonism impairs the acquisition of contextual fear, whereas administration into the BLA did not. Systemic D1R antagonism, on the other hand, was not found to impair the reconsolidation or extinction of contextual fear. These findings are novel and advance the current literature on the role of D1Rs during contextual fear learning and memory. This research aids in understanding the underlying causes of anxiety disorders such as PTSD and could shed light on why some people may be more susceptible to the development of such disorders depending on the modulation of fear learning by D1Rs. This could potentially be useful in the development of preventative therapies. Given that D1Rs were not found to have a role in contextual fear reconsolidation and extinction, which could be targeted for treatment, it would be advisable that future research pursues alternative hypotheses, for example, investigating the role of D2Rs in contextual fear reconsolidation and extinction.

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