ROLE OF 5-HT_6 RECEPTOR IN CONDITIONED LEARNING AND MEMORY

Susie Woods, BSc.

THESIS SUBMITTED TO THE UNIVERSITY OF NOTTINGHAM FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

School of Biomedical Sciences
July 2011

Abstract

The recently discovered 5-HT_6 receptor has generated interest due to increasing evidence for its role in feeding, obesity, anxiety, depression and cognition. Initial studies utilising selective 5-HT_6 receptor antagonists found pro-cognitive effects in various cognitive paradigms. In the last three years selective 5-HT_6 receptor agonists have been developed and initial reports suggested they impaired cognition as predicted, but more recent reports have found paradoxical pro-cognitive effects in learning and memory tasks. The main aim of the current thesis was to determine the role of the 5-HT_6 receptor in a conditioned emotion response (CER) task in rats. Both the effects of 5-HT_6 receptor antagonists and agonists given alone, and their abilities to reverse a cholinergic- or glutamatergic-induced memory impairment were analysed. Secondly, to analyse the intracellular mechanisms involved in the behavioural effects exerted following treatment with 5-HT_6 receptor ligands by examining changes in hippocampal protein expression.

Pre-treatment with either the muscarinic receptor antagonist, scopolamine, or the NMDA receptor antagonist, MK-801, induced memory impairment in the 24 hour retention trial. Post-training administration of 5-HT_6 receptor antagonist, SB-271046, and agonists, EMD 386088 and E-6801, had little effect on CER-induced behaviour when given alone, but both reversed the cholinergic- and glutamatergic-induced deficits.

Western blot analysis revealed no significant difference between hippocampal BDNF and 5-HT_6 receptor protein levels following any drug or shock treatment,

but some interesting trends were observed. CER slightly increased BDNF expression, this was reduced by scopolamine and MK-801 which in turn was reversed with SB-271046 and EMD 386088. CER decreased 5-HT_6 receptor expression, scopolamine caused further reduction, SB-271046 and EMD 386088 increased the expression following scopolamine. MK-801 increased 5-HT_6 receptor expression, whilst SB-271046 further enhanced this expression, EMD 386088 reduced it. No significant results were observed in the proteomic studies.

These findings provide further evidence for the exciting potential therapeutic use of 5-HT_6 receptor compounds in the treatment of cognitive dysfunction.

Publications

Poster

Woods, S. W., Clarke, N. N., Topham, I. A., Layfield, R., Fone, K. C. F. (2008). Do 5-HT_6 receptor antagonists alter emotional learning in a contextual fear conditioning paradigm? *Journal of Psychopharmacology* **S22**, 5, A14

Woods, S. W., Clarke, N. N., Topham, I. A., Layfield, R., Fone, K. C. F. (2008). Effects of 5-HT_6 receptor antagonist, SB-271046, on fear motivated learning and memory. *Serotonin Club Meeting*, **SCP020**.

Woods, S. W., Clarke, N. N., Layfield, R., Fone, K. C. F. (2009). Can a 5-HT_6 receptor agonist, EMD 386088, ameliorate a scopolamine-induced memory deficit in contextual fear conditioning? *British Neuroscience Association Abstract*., **20**, P39.05

Oral

Woods, S. W., Clarke, N. N., Layfield, R., Fone, K. C. F. (2009). Can 5-HT_6 receptor antagonists and agonists reverse impairments in hippocampal-dependent memory? *Short Oral Presentation, BAP Summer Meeting, Oxford, UK*

Acknowledgments

I would like to thank my supervisors Kevin Fone and Robert Layfield for their encouragement and support throughout my PhD. I would also like to thank the University of Nottingham and the Medical Research Council for their financial support. I am very grateful to GSK and Esteve for the kind gifts of the pharmacological compounds which made this work so interesting.

As I knew very little on arrival I have been taught a lot along the way at Nottingham, therefore I would like to thank friends in the lab, Liaque for Western blots (and the discussions), Ian K, Maddy, Dan and Dave for their advice on the models, and Ian T for his advice and help in every study. During first year I would have been a bit lost without Nola, so thanks to her for keeping me afloat.

A huge thanks to the girls, Irish, Warner and Welshy I would have gone insane a long time ago if it was not for you three and the good old gossips over tea, also for the advice and support you gave me, thank you. A bit further away, a big thanks to Daniella and Sam for always being on the other end of the phone when I needed you.

My biggest thanks has to go to my family, Mum, Dad, Lucy, Tom and Guy, for their love and support throughout my studies at Nottingham. Finally to my husband John, who has always been there at the end of the day, some longer than others! I want to thank you so much for listening to my rants (work and non-work related), cheering me up and having a constant supply of sweets, helping with my presentations and abstracts, basically for believing in me and always being there! For their constant love and support I would like to dedicate my thesis to my family and John.

Table of Contents

Table of Figures and Tables

Abbreviations and Pharmacological compounds

aa	Amino acid
AC	Adenylyl cyclase
ACh	Acetylcholine
AChEI	Acetylcholinesterase Inhibitor
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid
ANOVA	Analysis of variance
AO	Antisense oligonucleotides
AP5	NMDA antagonist: aminophosphonovaleric acid
APV	D,L-2-amino-5-phosphonovalerate
BBB	Blood brain barrier
BBXXB	Amino acid motif, B= basic residue and X= non basic residue
BDNF	Brain derived neurotrophic factor
BGC20-761	5-HT_6 receptor antagonist: 5-methoxy-2-phenyl-*N,N*-dimethyltryptamine
BLA	Basolateral amygdala
BMSU	Biomedical Sciences Unit
BS 5-OMe DMT	N_1-benzenesulfonyl-5-methoxy-*N,N*-dimethyltryptamine
BVT 5182	5-HT_6 receptor antagonist: 1-benzenesulfonyl-4-(piperazin-1-yl)-indole hydrochloride
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
5-CT	5-carboxamidotryptamine
CaMKII	Calcium-calmodulin dependent protein kinase II
cDNA	Complementary deoxyribonucleic acid
CER	Conditioned emotion response
CFC	Contextual fear conditioning
ChAT	Choline acetyltransferase
CNS	Central nervous system
COS-7	Cell line
CPP	3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid
CR	Conditioned response
CRE	cAMP response elements
CREB	cAMP response elements binding protein
CS	Conditional/ed stimulus
C-terminal	Carboxy terminal
DA	Dopamine
DH	Dorsal hippocampus
E-6387	5-HT_6 receptor agonist: 5-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)naphthalene-2-sulfonamide
E-6801	5-HT_6 receptor agonist: 6-chloro-N-(3-(2-(dimethylamino)ethyl)-1H-indol-5-yl)imidazo[2,1-b]thiazole-5-sulfonamide
EC50	Half maximal effective dose
ED	Extra-dimensional shift

EMD 386088	5-HT_6 receptor agonist: 5-chloro-2-methyl-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1H -indole hydrochloride
EMDT	5-HT_6 receptor agonist: 2-ethyl-5-methoxy-*N,N*-dimethyltryptamine
GABA	Gamma amino butyric acid
GAD-67	Glutamic acid decarboxylase
Glu	Glutamate
GPCR	Guanine nucleotide-binding protein coupled receptor
HEK-293	Human embryonic kidney (Cell line)
He-La	Cell line
5-HIAA	5-hydroxyindole acetic acid
HPA axis	Hypothalamic pituitary adrenal axis
5-HT	5-Hydroxytryptamine
5-HT_6-LI	5-HT_6 receptor like immunoreactivity
5-HTP	5-hydroxytryptophan
IA	Inhibitory avoidance
i.c.v	Intracerebroventricular
IC50	Half maximal inhibitory concentration
ID	Intra-dimensional shift
i.p	Intraperitoneal
ITI	Inter-trial interval
IUPHAR	International Union of Pharmacology
JEG-3	Cell line
kDa	Kilodaltons
LSD	Lysergic acid diethylamide
LTD	Long-term depression
LTM	Long-term memory
LTP	Long-term potentiation
LY-586713	5-HT_6 receptor agonist
MAP	Mitogen activated protein
MAO	Monoamine oxidase
MEST	Maximal electroshock seizure threshold
MK-801	NMDA receptor antagonist: (+)-5-methyl-10, 11-dihydro-5Hdibenzo[a, d]cyclohepten-5, 10-imine maleate,dizocilpine
mPFC	Medial pre-frontal cortex
mRNA	Messenger ribonucleic acid
MTL	Medial temporal lobe
MWM	Morris water maze
Na	Sodium
NBM	Nucleus basalis magnocellularis
NE	Norepinephrine
nM	Nano-molar
NMDA	*N*-methyl-$_D$-aspartic acid
NO	Nitric oxide
NOR	Novel object recognition
NS	Non-shocked
N-terminal	Amino terminal
PA	Passive avoidance
PAG	Periaqueductal gray

PCP	NDMA receptor antagonist: Phencyclidine
PCR	Polymerase chain reaction
pEC50	Negative logarithm of EC50
PFC	Prefrontal cortex
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PMDT	5-HT_6 receptor antagonist: 2-phenyl-5-methoxy-*N,N*-dimethyltryptamine
p.o	Oral administration
PPI	Pre-pulse inhibition
PRX-07034	5-HT_6 receptor antagonist: N-[1-(5-chloro-2,3-dimethoxyphenyl)ethyl]-2-methylsulfonyl-5-piperazin-1-ylaniline
6-OHDA	6-hydroxydopamine
R13*c*	5-HT_6 receptor agonist: (*R*)-2-chloro-*N*-(3-{[2R)-1-methyl-2-pyrrolidinyl]methyl}-1H-indol-5-yl)benzenesulfonamide
RA	Retrograde amnesia
Ro 04-6790	5-HT_6 receptor antagonist: 4-amino-*N*-(2,6-bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide
Ro 4368554	5-HT_6 receptor antagonist: 3-benzenesulfonyl-7-(4-methyl-piperazine-1-yl)-*H*-indole
Ro 63-0563	5-HT_6 receptor antagonist: 4-amino-*N*-(2,6 bis-methylamino-pyridin-4-yl)-benzene sulphonamide
RT-PCR	Real time polymerase chain reaction/Reverse transcription polymerase chain reaction
SB-258510	5-HT_6 receptor antagonist: N-[4-methoxy-3-(4-methyl-1-piperazinyl)-phenyl]-5-chloro-3-methylbenzo-thiophene-2-yl sulphonamide monohydrochloride
SB-258585	5-HT_6 receptor antagonist: 4-iodo-N-[4-methoxy-3-(4-methyl-piperazin-1-yl)-phenyl]-benzene sulphonamide
SB-271046	5-HT_6 receptor antagonist: 5-chloro-*N*-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide
SB-357134	5-HT_6 receptor antagonist: *N*-(2, 5-dibromo-3-fluorophenyl)-4-methoxy-3-piperazin-1-ylbenzenesulphonamide
SB-399885	5-HT_6 receptor antagonist: *N*-[3,5-dichloro-2-(methoxy)phenyl]-4-(methoxy)-3-(1-piperazinyl)benzenesulfonamide
SB-742457	5-HT_6 receptor antagonist: 3-benzenesulfonyl-8-piperazin-1-yl-quinoline trihydrochloride
SBP	Serotonin binding protein
SCH 23390	Dopamine 1 receptor antagonist
s.e.m	Standard error of the mean
SERT	Serotonin transporter
SH3	SRC homology 3 domain
SSRI	Selective serotonin reuptake inhibitor
STM	Short-term memory
TM	Transmembrane

TRP	Tryptophan
TTX	Tetrodotoxin
US	Unconditional/ed stimulus
WAY 181187	5-HT_6 receptor agonist
WAY 208466	5-HT_6 receptor agonist
WCST	Wisconsin card sorting task

1 General Introduction

The neurotransmitter 5-hydroxytryptamine (5-HT) has many peripheral and central functions consisting of gastrointestinal function, vascular constriction and dilation, platelet aggregation, regulation of appetite, sleep, mood, hallucination and pain. 5-HT acts upon several pharmacologically and structurally distinct 5-HT receptors, much research on the potential therapeutic effects of these receptors in treating anxiety, depression, migraine and cognition is ongoing. In the last decade the 5-HT_6 receptor has received a high level of interest as a potential target in the treatment of cognitive dysfunction in common central nervous system (CNS) disorders such as schizophrenia and Alzheimer's disease (AD).

The main aims of the current thesis were to optimise a conditioned emotion response (CER) paradigm in rodents, to utilise this to investigate the role of the 5-HT_6 receptor in this associative form of learning and memory by analysing the effects of ligands at the 5-HT_6 receptor on drug-induced deficits in this paradigm. Therefore this introduction will cover the background to the 5-HT_6 receptor and effects that ligands of this receptor have on learning and memory.

1.1 Cognition

Cognition is the term used to describe the collective mental process of learning and memory. Learning is the acquisition of new information, skill or knowledge,

and memory is the process of retaining and recalling this information. Learning can be classed as associative (forming connections between two or more stimuli), and non-associative (response to a repeated stimulus). Memory can be classed according to; type of information acquired (explicit, semantic or implicit), categorically (declarative or procedural, Figure 1-1), or the duration of which it lasts (short or long-term). An immense amount of research has been performed to try and understand the neural basis of learning and memory, and by combining psychology, behavioural neuroscience and neurobiology the circuits and molecular mechanisms involved in these processes have started to be identified (Milner et al., 1998; Thompson, 1986). It is known that declarative memory is the knowledge of events and facts; this relies upon the hippocampus, subiculum, and entorhinal cortex, whereas procedural memory is knowledge of skills and behaviours, and relies more heavily on the cerebellum and the basal ganglia (Lynch, 2004; Squire, 2004).

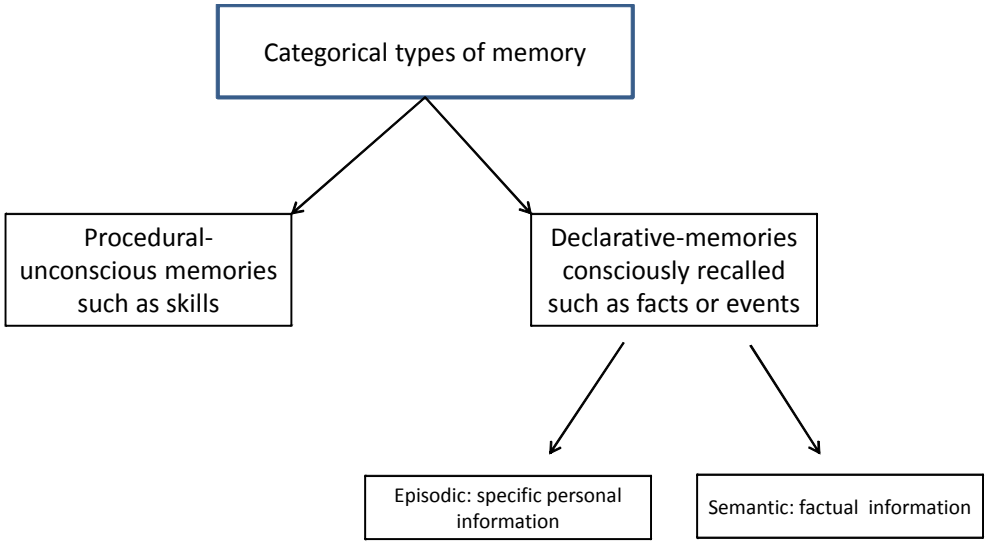


Figure 1-1-Schematic of categorical types of memory

Through lesioning and pharmacological manipulation the basic neuroanatomy and neurochemistry of cognition has been elucidated, with the medial temporal lobe (MTL) playing a crucial role in learning and memory (Suzuki and Eichenbaum, 2000). For instance, the frontal cortex plays a particularly important role in social recognition, and the medial prefrontal cortex (mPFC) is involved in attention and spatial learning (Dalley et al., 2004). The hippocampus is required for spatial and recognition memory, and hippocampal lesions have caused impairments in both memory types (Broadbent et al., 2004). The basolateral amygdala (BLA) is required for memory consolidation and projections from here to regions such as the hippocampus, caudate nucleus, nucleus basalis and cortex are crucial for this process (McGaugh, 2002). Furthermore, research has found that the amygdala aids acquisition, expression and extinction of fear memory (Ehrlich et al., 2009).

Functional imaging and lesioning studies in rodents have provided evidence that the neuronal circuitry involved in learning and memory is re-organised over a period of time (Bontempi et al., 1999; Wiltgen et al., 2004). Recently formed memories are stored temporarily within the structures of the MTL, and damage to this area leaves the subject incapable of forming new memories while remote memories remain intact (Milner et al., 1998; Nadel and Moscovitch, 1997; Scoville and Milner, 1957). In contrast, consolidated memories become independent of the MTL, with evidence proving they rely on neocortical circuits (Frankland et al., 2004a; Wang et al., 2006; Zolamorgan and Squire, 1990).

Cholinergic neurotransmission has been associated with learning and memory, evidence has found that a decline in cholinergic neurotransmission with old age and AD, suggesting a key role in memory impairment (Bartus et al., 1982; Coyle et al., 1983; Perry et al., 1992). Although improvements in cognition of patients suffering with mild to moderate AD occur following treatment with donepezil (an acetylcholinesterase inhibitor, AChEI), these are slight and transient and more effective drugs are required to provide sustained treatment for this progressive CNS disorder (Courtney et al., 2004). Studies have shown that treatment with AChEI causes a delay in long-term potentiation (LTP) decay within the hippocampus, providing evidence that increased cholinergic transmission positively modulates LTP and may be the underlying reason for pro-cognitive effects of these drugs (Barnes et al., 2000) (LTP is discussed further in Chapter 2). Lesions of the cholinergic neurones, which in turn decreases acetylcholine (ACh) levels, induces memory deficits and disrupts attention, further supporting this is the increase of ACh levels during pre-clinical behavioural paradigms (Pepeu and Giovannini, 2009).

Another neurotransmitter system associated with cognition is glutamate, the main excitatory neurotransmitter within the CNS. Disruptions of glutamate pathways occur in AD (Greenamyre and Young, 1989), supporting its role in cognition. The process of long-term potentiation (LTP), thought to be one possible mechanism for learning and memory, is dependent on *N*-methyl-$_D$-aspartic acid (NMDA) receptors (Bliss and Collingridge, 1993), further supporting the role of glutamate in cognition. In addition blockade of NMDA receptors impairs

memory in a variety of preclinical tasks, including fear conditioning (Csernansky et al., 2005b; Izquierdo et al., 1997; King et al., 2004).

Interactions between glutamatergic and cholinergic systems have been demonstrated in rodent behavioural studies, with glutamate receptor antagonists inducing deficits that are ameliorated with AChEI treatment (Csernansky et al., 2005b; Mikami et al., 2007). Memory deficits induced via antagonists of the NMDA and muscarinic receptors have been of similar magnitude in pre-clinical behavioural tests, supporting a role of these two neurotransmitter systems in learning and memory (Aigner, 1995).

1.2 Conditioned Emotion Response

CER is a form of Pavlovian conditioning, which studies associative learning. CER is often utilised to study learning and memory as subjects acquire the memory within one training session. Training involves pairing a non-aversive neutral stimulus, such as a context, or light and tone, the conditioned stimulus (CS), with an aversive stimulus, such as a foot shock the unconditioned stimulus (US), such that subsequent exposure to the CS induces a fear response which can be quantified and used as an index of learning and memory. Studies throughout this thesis extensively use CER to investigate the role of 5-HT_6 receptor ligands on learning and memory (see Chapter 2 for detailed methodology of CER).

1.3 5-Hydroxytryptamine

1.3.1 Discovery of 5-HT

Discovery of serotonin can be dated back as early as 1900-1910, researchers found an endogenous substance within clotted blood that induced vasoconstriction, although it was not discovered properly until 1948. During the 1930s Erspamer and colleagues were interested in smooth muscle contraction, and studied the properties of amines in this area, they discovered a novel substance in enterochromaffin cells of the gut that induced smooth muscle contraction, and named it enteramine (Erspamer, 1954). In 1948 the vasoconstrictor substance was isolated and characterised from bovine serum, and was named serotonin (Rapport et al., 1948a, b, c). The chemical structure of serotonin was determined and was found to be the indolealkylamine (chemically referred to as 3-(2-aminoethyl)indol-5-ol) or 5-hydroxytryptamine (5-HT, Figure 1-2-Structure of 5-HT, (Rapport, 1949)), and was chemically synthesised in the same year.

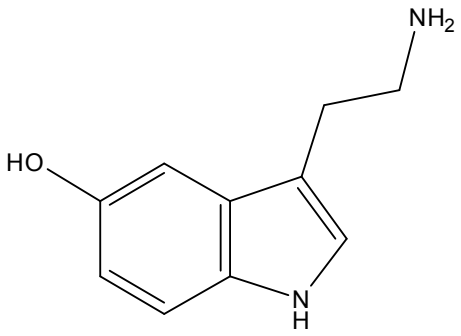


Figure 1-2-Structure of 5-HT

In 1952 it was found that enteramine was the same chemical substance that Rapport and colleagues had worked on, 5-HT (Erspamer and Asero, 1952). The function of 5-HT was still to be elucidated at this stage and the first evidence that 5-HT may act as a neurotransmitter was when 5-HT was found in the mammalian brain (Twarog et al., 1953). Due to the structural similarities between the potent hallucinogenic drug, lysergic acid diethylamide (LSD), and 5-HT, groups hypothesised that 5-HT may play a role in mental processing, which was supported with evidence that LSD could antagonise the effects of 5-HT (Shaw and Woolley, 1953; Woolley and Shaw, 1954). Following this it was shown that platelet serotonin levels were reduced with antidepressant treatment in patients (Marshall et al., 1960), and rat brain serotonin levels were decreased following treatment with the antipsychotic drug reserpine (Brodie et al., 1955; Pletscher et al., 1955; Whitaker-Azmitia, 1999).

1.3.2 Synthesis, storage, release and metabolism of 5-HT

The initial and rate-limiting step of 5-HT synthesis is the hydroxylation of the amino acid L-tryptophan which is an essential amino acid. Tryptophan is taken up by neurons via a plasma membrane transporter, hydroxylation occurs on the 5-postion by the enzyme tryptophan hydroxylase, to form 5-hydroxytryptophan (5-HTP), which in turn undergoes rapid decarboxylation, by the enzyme L-amino acid decarboxylase, to form 5-HT (Clark et al., 1954).

After biosynthesis 5-HT is stored in pre-synaptic nerve terminal vesicles with a serotonin binding protein (SBP) with high affinity. Upon depolarisation of the nerve terminal, an increase in the concentration of calcium (Ca^{2+}) induces the

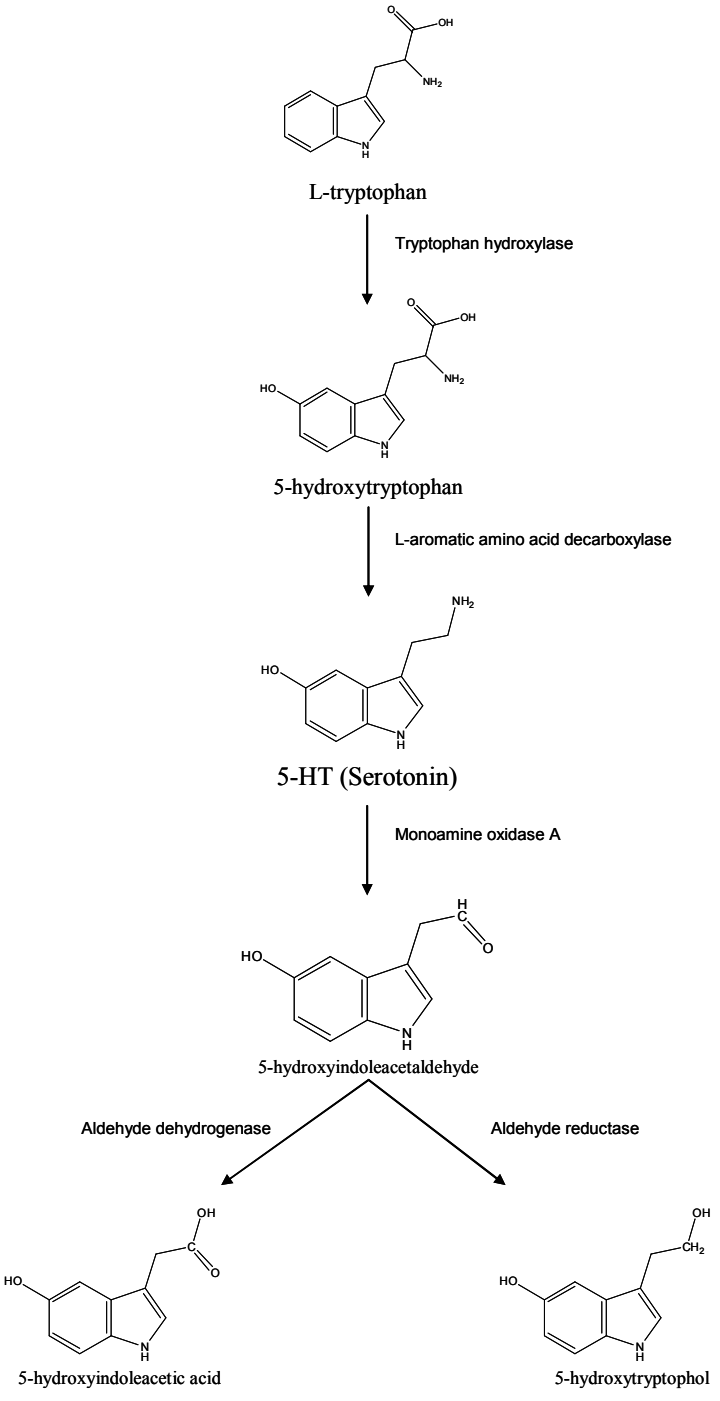


Figure 1-3-Schematic of synthesis of serotonin

release of the 5-HT vesicles which contain the SBP, via exocytosis, into the synaptic cleft (Elks et al., 1979; Tamir and Gershon, 1979).

The effects of serotonin within the synapse are terminated by its active reuptake via a sodium (Na^+)-dependent specific serotonin transporter (SERT, (Blakely et al., 1991). The degradation of 5-HT occurs in two steps, the initial step is oxidative deamination catalysed by monoamine oxidase (MAO) that forms 5-hydroxyindole acetaldehyde (Weissbach et al., 1961), secondly this metabolite is rapidly oxidised by aldehyde dehydrogenase to form the major metabolite 5-hydroxyindole acetic acid (5-HIAA, Figure 1-3**)** (Duncan and Sourkes, 1974). 5-HIAA is excreted in the urine and serves as a measure of 5-HT production within the body.

1.3.3 Anatomy of serotonergic system

As previously stated, the initial evidence for the presence of 5-HT within the brain was provided by Twarog and colleagues (1953). Following on from this, maps of serotonergic neurons and fibres within the brain were produced by application of formaldehyde-induced fluorescence against 5-HT. This method caused 5-HT to react with formaldehyde to produce a fluorescent compound 6-hydroxy-3,4-dihydro-β-carboline (5-hydroxytryptoline), which appeared yellow under ultraviolet light allowing the serotonergic neurons to be easily distinguished from catecholaminergic pathways. This initial technique found nine 5-HT containing cell groups (B1-B9) largely associated with the raphé nuclei (Figure 1-4, (Falck et al., 1962; Fuxe, 1965)). Methods of detection such as autoradiography following *in vivo* and *in vitro* application of [^{3}H]5-HT, and

immunohistochemistry for tryptophan hydroxylase and 5-HT itself have been utilised to map the serotonergic pathways in more detail in the brain (Azmitia and Segal, 1978; Steinbusch, 1981). The serotonergic cell bodies have been divided into the caudal (descending) and the rostral (ascending) system.

The caudal serotonergic system is comprised from descending axons primarily located in the medulla oblongata and caudal pons, consisting of the nucleus raphé pallidus (B1), nucleus raphé obscurus (B2) and nucleus raphé magnus (B3). These axons descend and innervate the spinal cord across different pathways. Cells from B3 terminate in laminae I and II of the dorsal horn gray matter, cells from B1 and B2 innervate the motor neurons of the ventral horn, particularly laminae VIII and IX, and finally projections innervate the pre-ganglionic neurons in the intermediolateral columns of the thoracic spinal cord (Bowker et al., 1982).

The rostral ascending serotonergic system is comprised of the median (B5 and B8) and dorsal (B6 and B7) raphé nuclei, as well as the caudal linear nucleus, the nucleus pontis oralis and supralemniscal region (B9), it is known to innervate the diencephalon, basal ganglia, limbic system and cortex.

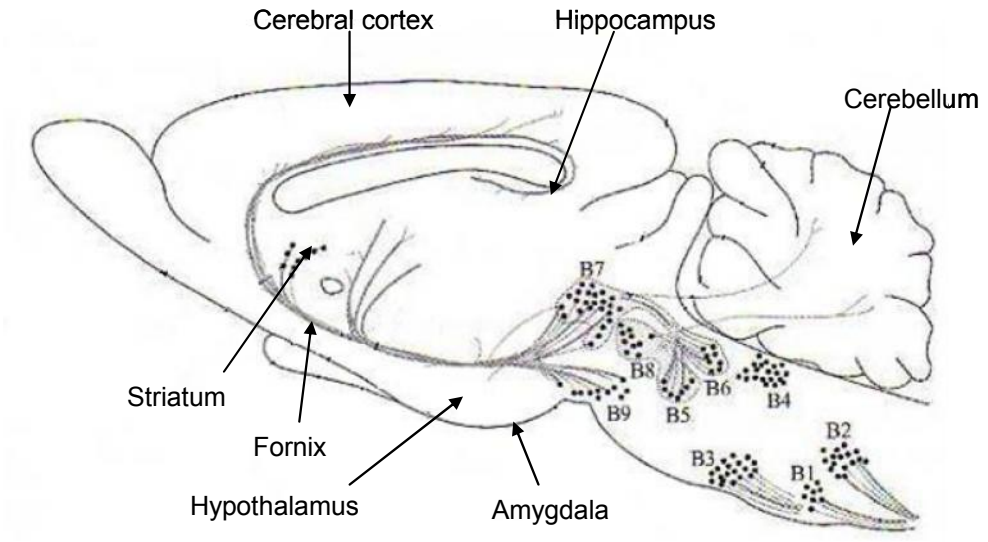


Figure 1-4- Schematic of main serotonergic projections within rat brain. The caudal serotonergic system consists of B1-B3, which are descending pathways innervating the spinal cord. The rostral ascending serotonergic system consists of B5-B9 and innervates the diencephalon, basal ganglia, limbic system and cortex (adapted from Cooper et al 2003).

1.3.4 Classification of 5-HT receptors

Initial studies that found multiple receptor subtypes for serotonin were performed by Gaddum and Picarelli (1957); the effect of 5-HT on contraction in isolated guinea-pig ileum tissue was analysed and 5-HT-induced contractions could be antagonised by either morphine or dibenzyline, neither of which are specific antagonists of 5-HT receptors. The serotonergic receptors were named M and D 5-HT receptors respectively, M receptors mediated depolarisation of cholinergic nerves and were sensitive to atropine and cocaine, D receptors mediated

contraction of smooth muscle and were susceptible to antagonism by LSD (Gaddum and Picarelli, 1957). No further classification of serotonin receptors were available for twenty years, until radioligand binding studies that utilised [^{3}H]5-HT, [^{3}H]LSD or [^{3}H]spiperone and identified two distinct 5-HT receptor subtypes within the cortex, that were designated 5-HT_1 and 5-HT_2 (Peroutka and Snyder, 1979). Different binding affinities of spiperone were identified at the 5-HT_1 therefore this was sub-divided into 5-HT_{1A} (nanomolar) and 5-HT_{1B} (micromolar) (Pedigo et al., 1981).

A new nomenclature of 5-HT receptors based on the ligand binding profile of receptors was proposed by Bradley and colleagues (1986) establishing three families, 5-HT_1-like, 5-HT_2 and 5-HT_3, containing five distinct receptors. 5-HT_1-like receptors had a high affinity for the agonist 5-carboxamidotryptamine (5-CT) and low affinity for the antagonists, methysergide and methiothepin, which were more potent at 5-HT_2 receptors. It was determined that 5-HT_2 receptors were identical to the D receptor that had been discovered in the guinea-pig ileum (Gaddum and Picarelli, 1957), they had a high affinity for the agonist α-methyl-5-hydroxytryptamine and antagonists, ketanserin, cyproheptadine and methysergide. The previously reported M receptor was re-named 5-HT_3 receptor, it was found to have a high affinity for the agonist 2-methyl-5-hydroxytryptamine and antagonists cocaine, MDL 72222 and ICS 205-930 (Bradley et al., 1986).

Due to the cloning of novel 5-HT receptors during the 1980s and 1990s this classification of serotonin receptors became outdated and the International

Receptor	CNS location	Neuronal location	Chromosome	Structure	Agonists	Antagonists	Functions
5-HT_{1A}	Hippocampus, lateral septum, raphé nuclei, and cortex	Pre- and post-synaptic	5q11.2–q13	421 aa	8-OH-DPAT, dipropyl-5-CT	(S)-UH-301, WAY 100 135, WAY 100 635, NAD 299	↑ NE and ACh, ↓ 5-HT and Glu. Hyperphagia, hypothermia, anxiolysis
5-HT_{1B}	Striatum, raphé nucleus, hippocampus	Pre- and post-synaptic	6q13	390 aa	L-694247, Ru 24969, 5-CT, CP 93129	SB-224289, SB-216641	↓ 5-HT and ACh. Hypophagia, hypothermia, penile erection, myoclonic jerks
5-HT_{1D}	Hippocampus, cortex, dorsal raphé nucleus, spinal cord and periaquaductal grey	Pre-synaptic	1p34.3-p36.3	377 aa	PNU 109291	BR-15572	↓ 5-HT and NE. Antimigraine
5-HT_{1E}	Cortex, hippocampus, amygdala, hypothalamus	Post-synaptic	6q14-q15	365 aa	n/d	n/d	n/d
5-HT_{1F}	Hippocampus, cortex, dorsal raphé nucleus, striatum, hypothalamus and thalamus	n/d	3p13-p14.1	366 aa	LY334370, LY344864, BRL 54443	n/d	May have a role as an autoreceptor. Visual and cognitive functions
5-HT_{2A}	Cortex, hippocampus, olfactory tubercle and striatum	Post-synaptic	13q14-q21	471 aa	DOI	MDL 100907	↓ NE. Hyperthermia, alter BDNF expression
5-HT_{2B}	Cerebellum, lateral septum, hypothalamus and medial amygdala	n/d	2q36.2-q37.1	481 aa	BW 723C86	RS 127445, SB-204741, SB 200646	Anxiety, hyperphagia, reduces grooming behaviour, vasorelaxation

5-HT_{2C}	Choroid plexus,cortex, hippocampus, nucleus accumbens, amygdala and basal ganglia	Post-synaptic and possibly pre-synaptic?	Xq24	458 aa	MK 212, Ro 60-0175	SB-242084, RS-102221	↑ NE and DA. Hypolocomotion, hypophagia, anxiety, penile erection and hyperthermia
5-HT_{3}	Hippocampus, area postrema, dorsal motor nucleus of the solitary tract	Pre-synaptic? Post-synaptic	11q23.1-q23.2	487 aa for 3A subunit 441 aa for 3B subunit	PBG	MDL 72 222, ondansetron, granisetron, tropisetron	↑ 5-HT, GABA, DA and CCK. ↓ ACh. Cognition, anxiety, psychosis
5-HT_{4}	Striatum, Hippocampus, amygdala, olfactory tubercle		5q31-q33	Splice variants: A 387 aa B 388 aa C 380 aa D 360 aa E 378 aa	BIMU-8, RS 67506, ML 10302	GR 113808, SB-204070, RS 39604	↑ 5-HT, ACh and DA . Cognition, anxiety
5-HT_{5A}	Cerebellum, hippocampus, hypothalamus, striatum, cerebral cortex.		7q36.1	357 aa	n/d	n/d	n/d
5-HT_{5B}	Cortex, hippocampus, dorsal raphé nucleus, olfactory tubercle	n/d	2q11-q13	370 aa	n/d	n/d	n/d
5-HT_{7}	Thalamus, hypothalamus, hippocampus	Post-synaptic	10q23.3-q24.3	445 aa	n/d	SB 269970, SB 258719	Circadian rhythms, seizures

Table 1-1- Summary of 5-HT receptors (except 5-HT_6 receptor). All receptors are GPCRs, except the 5-HT_3 receptor which is an ion channel. More detailed review in Hoyer et al 1994 (n/d-not determined, NE-norepinephrine, ACh-acetylcholine, DA-dopamine, Glu-glutamate).

Union of Pharmacology (IUPHAR) created a new nomenclature system for 5-HT receptors that included the operational, transductional and structural characteristics of each receptor (Hoyer et al., 1994; Humphrey et al., 1993).

To date there are seven 5-HT receptor families, 5-HT_{1-7}, consisting 14 structurally and pharmacologically distinct subtypes (Table 1-1, (Barnes and Sharp, 1999; Hoyer et al., 1994). As work in the current thesis focuses on the 5-HT_6 receptor more detail of this particular receptor will be reviewed.

1.3.5 5-HT_6 receptors

1.3.5.1 Molecular biology

Two independent research groups successfully isolated and identified the 5-HT_6 receptor in the rat brain using molecular biology techniques rather than pharmacological profiling, although their results were not identical (Monsma et al., 1993; Ruat et al., 1993). Polymerase chain reaction (PCR) technique was used to amplify the cDNA sequence of two degenerate primers, derived from the third and sixth transmembrane (TM) region of a previously cloned guanine nucleotide-binding protein coupled receptor (GPCR), from mRNA purified from rat striatum (Monsma et al., 1993). The encoded protein had 437 amino acid (aa) residues, and had a molecular mass of 46.8 kDa (Monsma et al., 1993). Ruat and colleagues (1993) used a slightly different technique and utilised a nucleotide probe from a rat $histamine_2$ (H2) receptor to screen the cDNA taken from rat striatum to encode the functional 5-HT_6 receptor. This group found the clone to be a 436 aa protein sequence, with a molecular mass of 46.92 kDa. Both groups

identified seven hydrophobic areas, and significant homology with other 5-HT receptors, the sequence was found to contain an intron within the third intracellular loop (Monsma et al., 1993; Ruat et al., 1993).

Due to the differences in drug affinities between species and the interest in 5-HT_6 receptors as a tool for psychopharmacology the human 5-HT_6 receptor was cloned and characterised using PCR amplification from the human caudate cDNA library (Kohen et al., 1996). The human 5-HT_6 receptor protein consists of 440 aa residues, with a molecular mass of 46.96 kDa. Hybridisation studies mapped the human 5-HT_6 receptor gene to chromosome 1p35-p36. Hydropath analysis found seven hydrophobic regions which, like the rat 5-HT_6 receptor, were predicted to be transmembrane (TM) regions. The reading frame of the receptor contains two introns, found within the third intra- and extra-cellular loops, and comparison between the cDNA and genomic sequence of the human 5-HT_6 receptor identified a silent base exchange producing an *Rsa*1 polymorphism at position 267. Initial comparison studies between the human 5-HT_6 and the published rat 5-HT_6 cDNA sequences (Monsma et al., 1993; Ruat et al., 1993) revealed a frame shift, and re-sequencing the rat 5-HT_6 cDNA sequence highlighted a frame shift error between 1030 and 1040 base pairs. The corrected rat 5-HT_6 receptor protein is a linear 438 aa chain with a molecular mass of 46.81 kDa. The human 5-HT_6 receptor aa sequence had 89% homology with the corrected rat sequence, and their nucleic acid sequences shared 85% homology (Kohen et al., 1996). The mouse 5-HT_6 receptor gene was successfully identified following PCR amplification of primers taken from mouse brain cDNA (Kohen et al., 2001). The protein was found to be a 440 aa

polypeptide, with a molecular mass of 47 kDa. Comparison studies found the nucleic acid sequence of the mouse 5-HT_6 receptor to have 94% and 84% homology to rat and human respectively, and 97% and 89% aa sequence homology with rat and human receptors accordingly (Kohen et al., 2001).

The structure of the 5-HT_6 receptor is characterised by a short third intracellular loop, approximately 50 aa, and a long C-terminus, approximately 120 aa, both of these are traits of a GPCR (Lefkowitz and Caron, 1988). Due to the abundance of threonine and serine residues found in both these areas there are many potential phosphorylation target sites (Monsma et al., 1993; Ruat et al., 1993). Constitutive activity of a receptor can be induced by mutations of the BBXXB motif (B=basic and X=non-basic protein residues) located within an intra-cellular loop of many GPCR including 5-HT_6 receptors. Kohen and colleagues (2001) reported constitutive activity of wild-type mouse receptors expressed in both JEG-3 and COS-7 cells, this increased with the level of expression in cells, the study utilised a reporter gene assay rather than direct measurement of cyclic adenosine monophosphate (cAMP). Three of five point mutations within the BBXXB motif caused a reduction in constitutive activity, K264, K267 and A268. Due to the sequence homology between species, it was suggested that rat and human 5-HT_6 receptors may elicit a similar behaviour.

Characteristics of the 5-HT_6 receptor which distinguish it from the other G protein-coupled 5-HT receptors is the consensus *N*-linked glycosylation site on the amino terminal tail, Asn^{9} or Asn^{10}, found upon the highly conserved cysteine residues (Cys^{99}-Cys^{180}), thought to involve a disulphide bond between the first

and second extracellular loops. Another distinctive structural feature of the 5-HT_6 receptor was the leucine zipper motif found present at the end of the third TM region (Leu^{115}-Leu^{136}, (Kohen et al., 1996; Monsma et al., 1993; Ruat et al., 1993).

1.3.5.2 Ligand Binding sites

Synthesising mutant receptors via site-directed mutagenesis has identified key residues within 5-HT_6 receptor that are critical for the ligand binding domain. Two key residues have been identified on many bioamine receptors to play a role in ligand binding, on the 5-HT_6 receptor the residue at position 196 is a threonine, mutating this to alanine caused a 10- and 6-fold reduction in affinity for LSD and 5-HT, and reduced potency of these agonists to stimulate adenylyl cyclase (AC). Findings indicate that Thr196 on wild-type 5-HT_6 receptors forms a hydrogen bond with the indole nitrogens on tryptamines and N_1-unsubstituted ergolines (Boess et al., 1997). The same group identified another key residue, Aspartate106 replacement with aspargine (D106N), on TM III, abolished [^{3}H]-LSD binding to receptor expressed in HEK-293 cells. Functional studies found that the D106N mutant significantly reduced agonist activity, with a 3,600- and 500-fold decrease of AC accumulation following 5-HT and LSD. Mutation of tryptophan, at position 102, with phenylalanine (W102F) caused small but significant reductions in affinity and potency of most ligands, except ritanserin and metergoline which increased. A double mutation of neighbouring residues, Ala287 and Asn288 with leucine and serine (A267L, N288S), caused an increase in affinity for sumatriptan and ergopeptine ligands (Boess et al., 1998a). Within human 5-HT_6 receptor different modes of binding for 5HT and antagonist

arylsulfonyltryptamine were determined, with the former binding between TM domains III and VI and the latter between VI, VII and I. Residues found to play a role in ligand binding were Val^{107}, Cys^{110} and Ser^{111} on TM III, Ala^{192} and Ser^{193} on TMV, and Phe^{284}, Phe^{285} and Asn^{288} on TMVI (Pullagurla et al., 2004).

Utilising a computer modelling system Hirst and colleagues (2003) aligned the aa sequences of the mouse, rat and human 5-HT_6 receptors to determine differences between species. Four aa residues were identified within the mouse sequence that differed from those in other two species, these were serine175 within second extracellular loop, tyrosine188 in TM5, serine277 and serine290 in TM6, which correlated to praline175, phenylalanine188, glycine275 and aspargine288 in rat and human sequence. In particular Try188 and Ser290 were found to have the greatest effect on affinity for ligands, therefore these residues may underlie the differences in binding affinities observed between species (Hirst et al., 2003).

1.3.5.3 Signal Transduction

When expressed in HEK-293 or COS-7 cells the rat 5-HT_6 receptor was positively coupled to cAMP production, it was also shown that a 5-fold increase in accumulation was produced with administration of 5-CT and 5-methoxamidotryptamine (Monsma et al., 1993; Ruat et al., 1993). Analogous to this the human 5-HT_6 receptor transfected in He-La cells was found to be positively coupled to AC, with administration of 5-HT causing a 2.5 to 8-fold increase of cAMP accumulation (Kohen et al., 1996).

The signal transduction mechanism coupled to the 5-HT_6 receptor *in vivo* is still yet to be elucidated, a recent study used a yeast two-hybrid assay with the C-terminus of the human 5-HT_6 receptor identified an interaction with Fyn-tyrosine kinase following a screen of the human cDNA library (Yun et al., 2007). Following this it was determined the interaction was mediated via the SH3 domain of Fyn in two cell lines and the rat adult brain. Immunohistochemistry found co-localisation of Fyn and 5-HT_6 receptors within the hippocampus, cortex and hypothalamus in the rat brain. Functional assays identified the interaction between Fyn and 5-HT_6 receptors was found be reciprocal, as expression of Fyn increased 5-HT_6 receptor activity with increased expression, and activation of the receptor with 5-HT caused an increase in Fyn phosphorylation (Yun et al., 2007). Interestingly 5-HT_6 receptors activated Erk-1/2, via Ras and MEK-dependent pathways in cells, which was decreased in the presence of Fyn inhibitor PP2 providing evidence for a role of Fyn in 5-HT_6 receptor signalling (Yun et al., 2007).

1.3.5.4 Distribution of 5-HT_6 receptors

Northern blot analysis, *in situ* hybridisation and real-time-PCR (RT-PCR) found the 5-HT_6 receptor mRNA in striatum, nucleus accumbens, hippocampus, amygdala, cerebral cortex, hypothalamus, thalamus, caudate nucleus and putamen of rat and human brain samples (Boess et al., 1998b; Kohen et al., 1996; Monsma et al., 1993; Ruat et al., 1993; Yoshioka et al., 1998). Levels of 5-HT_6 receptor mRNA were detected in rat spinal cord with RT-PCR (Gerard et al., 1996) but not *in situ* hybridisation studies (Ward et al., 1995). Within the periphery there have been contradictory reports, some groups have failed to find

any expression in rat or human heart, liver, lung, skeletal muscle, kidney, pancreas, spleen, small intestine, placenta, testis, stomach, prostate or uterus (Hirst et al., 2003; Monsma et al., 1993) whereas others have found faint expression in the rat stomach and pig adrenal glands (Ruat et al., 1993). Due to the limited 5-HT_6 mRNA expression within the periphery, ligands that act upon this receptor have the potential to have limited peripheral side effects if given therapeutically.

To elucidate the distribution of 5-HT_6-receptor-like immunoreactive (5-HT_6-LI) material within the brain polyclonal antibodies were raised against the C-terminal of the 5-HT_6 receptor (Gerard et al., 1997; Hamon et al., 1999). Under low magnification light microscopy the distribution of 5-HT_6 receptors was found to generally match that observed with the transcript, except that 5-HT_6 mRNA was found in hypothalamus, habenula and raphé nuclei (Gerard et al., 1996) and these areas elicited undetectable 5-HT_6-LI. Utilising a high magnification light microscope of 5-HT_6 receptors were localised, within the hippocampus, in the strata oriens, radiatum of CA1 and molecular area of dentate gyrus, with no staining observed on the pyramidal or granular cells, where mRNA has been localised. These findings indicate that 5-HT_6 receptors may be transported from pyramidal and granular cells to dendritic areas. Electron microscopy found 5-HT_6-LI in striatum and hippocampus to be associated with dendrites which were making synapses with non-labelled axons. The discrepancies between localisation studies of the transcript and protein could be due to variations in efficiency in transduction and/or translation of 5-HT_6 receptor gene, techniques not being sensitive enough to detect low levels, or addressing of the 5-HT_6

receptor protein at a distance from its site of synthesis. Regional distribution of 5-HT_6-LI generally matched that of 5-HT_6 mRNA suggesting the receptor is expressed in close proximity to the site of synthesis, such as somas and/or dendrites (Gerard et al., 1997; Hamon et al., 1999).

Specific binding of the 5-HT_6 receptor antagonist [^{125}I]-SB-258585 was used to determine protein levels of 5-HT_6 receptor within rat and human brain tissue, corresponding to the levels of receptor mRNA, high levels were identified in the striatum, nucleus accumbens, cerebral cortex, hippocampus, caudate nucleus and putamen. In contrast to this, very low levels of 5-HT_6 receptor levels were found in mouse brain regions (Hirst et al., 2003), but still detected in the striatum, nucleus accumbens and cortex of mice brains (Svenningsson et al., 2007).

Levels of 5-HT_6 receptor mRNA were not altered with 5,7-dihydroxytryptamine-induced lesions of serotonergic neurons, which caused a 90% reduction in SERT mRNA, within the anterior raphé area of rat brains, indicating it is not a pre-synaptic autoreceptor (Gerard et al., 1996). *In situ* hybridisation studies found the 5-HT_6 receptor mRNA within serotonergic projection fields and not serotonergic-containing cell bodies suggesting that the receptor was post-synaptic (Ward et al., 1995).

Studies illustrated that 5-HT_6 receptors are not located on cholinergic neurons, initial evidence was provided with dual labelled immunohistochemistry, which utilised an N-terminal directed antibody for the 5-HT_6 receptor, found very little co-localisation of 5-HT_6 receptors and choline acetyltransferase (ChAT,

(Woolley et al., 2004)). This finding was further supported with evidence from Marcos and colleagues (2006), a selective lesion of the cholinergic system, induced via an injection of the immunotoxin 192-IgG-saporin into the nucleus basalis magnocellularis (NBM), caused a significant reduction of AChE and ChAT levels, but no effect on 5-HT_6 receptor mRNA or protein levels, nor did it affect the binding of [^{125}I]-SB-258585 in the frontal cortex (Marcos et al., 2006). Following this dual-labelling immunohistochemical analysis found co-localisation of the 5-HT_6 receptor with the neuronal form glutamic acid decarboxylase (GAD67), providing evidence of receptor expression on GABAergic neurones (Woolley et al., 2004).

1.3.5.5 Pharmacology of the 5-HT_6 receptor

The pharmacology of rat, human and mouse 5-HT_6 receptor has been investigated *in vitro* using [^{125}I]-LSD, [^{3}H]-LSD, [^{3}H]-5-HT, [^{3}H]-Ro-63-0563, [^{125}I]-SB-258585 binding. Transfected HEK-293, COS-7 cells elicited high affinity for [^{3}H]-LSD and [^{125}I]-LSD, and 5-HT was the only biogenic amine capable of fully inhibiting binding. The receptor also elicited high affinity for the antipsychotics clozapine and loxapine and the tricyclic antidepressants amoxipine, clomiprimine and amitriptyline (Hirst et al., 2003; Kohen et al., 1996; Ruat et al., 1993).

Studies have demonstrated that there are pharmacological differences between the mouse 5-HT_6 receptor and the rat and human 5-HT_6 receptor. Affinity for 5-HT differed slightly between species, a significant 24- and 1900-fold decrease in affinity for the antagonists SB-357134 and Ro 04-6790 was observed in mouse 5-HT_6 receptors compared to both rat and human receptors (Hirst et al., 2003). SB-

271046 was found to have a lower affinity for the 5-HT_6 receptor in the mouse compared with human or rat, and Ro 04-6790 did not bind to the mouse 5-HT_6 receptor (Lindner et al., 2003). This study clearly demonstrates that rats should be used to test the therapeutic effects of 5-HT_6 receptor compounds, as effects observed in mouse models will not correlate as well to the effects in humans.

1.3.5.6 5-HT_6 receptor antagonists

Roche characterised the first potent and selective 5-HT_6 receptor antagonists, 4-amino-N(2,6 bis-methylamino-pyrimidin-4-yl)-benzene sulphonamide (Ro 04-6790) and 4-amino-N(2,6 bis-methylamino-pyridin-4-yl)-benzene sulphonamide (Ro 63-0563, Sleight et al 1998). Binding assays with [^{3}H] LSD demonstrated that both Ro 04-6790 and Ro 63-0563 displayed high affinities for the rat, pK_i values of 7.35 and 7.83, and human, pK_i values of 7.26 and 7.91, 5-HT_6 receptors. Ro 04-6790 was highly selective (>100-fold) for 5-HT_6 receptors versus 23 other binding sites, Ro 63-0563 also exhibited high selectivity for 5-HT_6 receptors (>100-fold versus 69 binding sites), but had moderate affinity for 5-HT_{2A} (pK_i=5.32) and 5-HT_{2C} (pK_i =5.69) receptors. Both compounds were found to be competitive antagonists at the human 5-HT_6 receptor with pA_2 values of 6.75 and 7.1 for Ro 04-6790 and Ro 63-0563 respectively, this was supported by no effect on basal cAMP accumulation in cells.

Ro 04-6790, but not Ro 63-0563, was found to cross the blood brain barrier (BBB), with concentrations within the cerebrospinal fluid that would occupy 70% of receptors, indicating Ro 04-6790 was a better candidate for investigations into the functional role of 5-HT_6 receptors in CNS (Sleight et al., 1998).

The following antagonists to be discovered for the 5-HT_6 receptor were the SmithKline Beecham compounds, initially 5-chloro-*N*-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide (SB-271046, (Bromidge et al., 1999; Routledge et al., 2000)) and *N*-(2,5-dibromofluorophenyl)-4-methoxy-3-piperazin-1-ylbenzenesulfonamide (SB-357134, (Bromidge et al., 2001; Stean et al., 2002)). Studies found SB-271046 and SB-357134 to have high affinity for human 5-HT_6 receptors with pK_i 8.9, and 8.5, both compounds displaced [^{125}I]-SB-258585 from human caudate putamen, rat and pig striatal membranes with p*Ki* values of 8.81 and 8.22, 9.02 and 8.44, and 8.55 and 8.61 accordingly. SB-271046 and SB-357134 are highly selective for the 5-HT_6 receptor, with >200-fold sensitivity over all other serotonin receptors and other receptors, enzymes and ion channels. Pharmacological studies found SB-271046 and SB-357134 were competitive antagonists, pA_2 values of 8.7 and 7.63, CNS penetration of 10% and 19%, with low blood clearance, 7.7 and 14 ml min^{-1} kg^{-1}, and both compounds have good oral bioavailability 82% and 65%. SB-271046 has a half-life of 4.8 hours in rats (Bromidge et al., 2001; Routledge et al., 2000; Stean et al., 2002).

Hirst and colleagues characterised the selective radioligand [^{125}I]-SB-258585 (Hirst et al., 2000), and found this to bind to transfected He-La cells with sub-nanomolar affinity, the levels of specific binding of this radioligand to rat and pig striatal membranes and human caudate putamen were 59.7, 65.5 and 67.5% accordingly, which is much greater than that observed with [^{3}H]-Ro 63-9563 (Boess et al., 1998b).

Work was performed on 2-substituted tryptamines as possible ligands at the 5-HT_6 receptor, this identified 2-phenyl-5-methoxy-*N,N*-dimethyltryptamine (PMDT). PMDT displayed good affinity, pK_i=20nM, although had low affinities for 5-HT_{2A} and 5-HT_7 receptors as well, 470 and 155nM, in a functional assay PMDT elicited antagonist properties with inhibition of AC (Glennon et al., 2000). Studies identified N_1-(benzenesulfonyl)tryptamines as novel 5-HT_6 receptor antagonists, with N_1-benzenesulfonyl-5-methoxy-*N,N*-dimethyltryptamine (BS 5-OMe DMT) displaying high affinity for human 5-HT_6 receptors, pK_i of 2.3nM, with affinity for the 5-HT_{2A} and 5-HT_{2C} receptors as well, pK_i=130 and 23nM accordingly. BS 5-OME DMT acted as an antagonist, with a pA_2 value of 8.88nM (Russell et al., 2001; Tsai et al., 2000).

Roche performed experiments on *N*-heteroaryl and *N*-arylbenzenesulfonamide analogues to determine the binding affinities at 5-HT_6 receptors. Optimal compounds synthesised were 4-amino-N-(6-methylamino-2-pyrrolidin-1-yl-pyrimidin-4-yl)-benzene sulphonamide (6r) and 4-amino-N-(3-methylamino-5-trifluoromethylphenyl)-benzene sulphonamide (20c), with pK_i values of 7.1 and 7.5, and log*D* values of 2.44 and 2.0 (indicative of brain penetration) accordingly (Bos et al., 2001). Riemer described the synthesis and pharmacology of 4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine, a potent (pK_i=9) antagonist, found to be over 100-fold selective for 5-HT_6 receptors, it is a potent antagonist characteristics (pA_2=8.5), with a half-life of 3-3.5 hours, good oral bioavailability, 46%, and a brain to plasma ratio of 24% (Riemer et al., 2003).

N_1-arylsulfonyl-3-(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole derivatives were found to be potent and selective antagonists, with two compounds screened found to have 0.4 and 3.0nM affinity for the receptor and antagonising production of AC at sub-nanomolar concentrations (Cole et al., 2005a). The same year Cole and colleagues identified the 5-arylsulfonylamido-3-(pyrrolidin-2-ylmethyl)-1*H*-indoles as 5-HT_6 receptor ligands, this study found the (*R*)-enantiomers to act as agonists (see section 1.3.5.7) and (*S*)-enantiomers to have moderate antagonist activity (Cole et al., 2005b).

Recent literature has described the pharmacokinetic (PK) and pharmacodynamic activity of SB-399885 (*N*-[3,5-dichloro-2-(methoxy)phenyl]-4-(methoxy)-3-(1-piperazinyl)benzenesulfonamide), a high affinity 5-HT_6 receptor antagonist for recombinant and native human 5-HT_6 receptors with pK_i values of 9.11 and 9.02 respectively, it also has a pK_i of 8.81 for the rat 5-HT_6 receptor. It was found to be a competitive antagonist, pA_2 of 7.85, and over 200-fold sensitivity for the 5-HT_6 receptor. SB-399885 has good oral bioavailability (52%), with a long duration of action and the PK data correlated with the results from maximal electroshock seizure threshold (MEST) tests with peak brain concentration reached 4 hours post-dose, and peak blood concentration 3 hours post-dose. The half-life of SB-399885 is 2.2 hours (Hirst et al., 2006). These results indicate that SB-399885 is superior to SB-271046 and Ro 04-6790 as a selective 5-HT_6 receptor antagonist.

Recently work has identified Ro 4368554 (3-benzenesulfonyl-7-(4-methyl-piperazine-1-yl)-*H*-indole), as a novel, selective (>50-fold compared to other

Ligand Name (Antagonists)	Affinity	Selectivity	Competitive antagonism	Additional information
Ro 04-6790 [1]	pK_i=7.35 and 7.26 for rat and human receptors	>100-fold versus 23 other binding sites	pA_2=6.75. No effect on basal cAMP	Cross the BBB. Concentrations within CSF that occupies 70% of receptors.
Ro 63-0563 [1]	pK_i=7.83 and 7.91 for rat and human receptors	>100-fold versus 69 binding sites. Has pK_i=5.32 and pK_i =5.69 values for 5-HT_{2A} and 5-HT_{2C} receptors	pA_2=7.1. No effect on basal cAMP	-
SB-271046 [2, 3]	pK_i=8.9 for human receptors	>200-fold versus all other serotonin receptors and other receptors, enzymes and ion channels	pA_2=8.7	CNS penetration of 10%. Low blood clearance 7.7 ml min^{-1} kg^{-1}. Good oral bioavailability 82% Half-life = 4.8 hours in rats
SB-357134 [4, 5]	pK_i=8.5 for human receptors	>200-fold versus all other serotonin receptors and other receptors, enzymes and ion channels	pA_2=7.63	CNS penetration of 19%. Low blood clearance 14 ml min^{-1} kg^{-1} Good oral bioavailability 65%.
PMDT [6]	pK_i=20nM for human receptors. Low affinity for 5-HT_{2A} (470 nM) and 5-HT_7 (155nM) receptors	-	Inhibited AC	-
BS 5-OMe DMT [7,8]	pK_i of 2.3nM for human receptors. Affinity for the 5-HT_{2A} (130nM) and 5-HT_{2C} (23nM) receptors	-	pA_2=8.88nM	-
4-amino-N-(6-methylamino-2-pyrrolidin-1-yl-pyrimidin-4-yl)-benzene sulphonamide (6r) [9]	pK_i= 7.1	-	-	log$D$=2.44 (indicative of brain penetration)

4-amino-N-(3-methylamino-5-trifluoromethylphenyl)-benzene sulphonamide (20c) [9]	pK_i= 7.5	-	-	log$D$= 2.0 (indicative of brain penetration)
4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine [10]	pK_i=9	>100-fold	pA_2=8.5	Good oral bioavailability 46% Half-life=3-3.65 hours Brain to plasma ratio 24%
SB-399885 [11]	pK_i=9.11 and 9.02 for recombinant and native human receptors. pK_i=8.81 for rat receptor	>200-fold	pA_2=7.85	Good oral bioavailability 52% Half-life=2.2 hours
Ro 4368554 [12]	pK_i=9.4	>50-fold	-	-

Table 1-2- Overview of 5-HT$_6$ receptor antagonists and their properties.

[1] Sleight et al., 1998. [2] Bromidge et al., 1999. [3] Routledge et al., 2000. [4] Bromidge et al., 2001. [5] Stean et al., 2002. [6] Glennon et al., 2000. [7] Russell et al., 2001. [8] Tsai et al., 2000. [9] Bos et al., 2001. [10] Riemer et al., 2003. [11] Hirst et al., 2006. [12] Schreiber et al., 2007.

targets) 5-HT_6 receptor antagonist which displays high affinity for the receptor (pK_i of 9.4) and good brain penetrance (0.8-1.1 brain/plasma ratio, (Schreiber et al., 2007)).

1.3.5.7 5-HT_6 receptor agonists

Development and synthesis of 5-HT_6 receptor agonists has been more difficult than 5-HT_6 receptor antagonists due to the problem of achieving specificity. One of the first agonists to be identified was 2-ethyl-5-methoxy-*N,N*-dimethyltryptamine (EMDT), which was found to a have similar affinity for human 5-HT_6 receptors as 5-HT (K_i=16nM and 75nM, accordingly). EMDT was not entirely selective for the 5-HT_6 receptor and displayed considerable affinity for 5-HT_{1A} (K_i=170nM), 5-HT_{1D} (K_i=290nM) and 5-HT_7 (K_i=300nM), with only 10-20 fold selectivity. EMDT activated AC, behaving as a full agonist at 5-HT_6 receptors, and displayed similar potency to 5-HT (ED_{50}=3.6 and 5.0 respectively, (Glennon et al., 2000)).

As previously mentioned, 5-arylsulfonylamido-3-(pyrrolidin-2-ylmethyl)-1*H*-indoles have been synthesised and a series of derivatives tested for efficacy at the 5-HT_6 receptor, the optimal candidate from this study was (*R*)-2-chloro-*N*-(3-{[2R)-1-methyl-2-pyrrolidinyl]methyl}-1H-indol-5-yl)benzenesulfonamide (R-13c) with a K_i of 1nM, and an EC_{50} value of 1.1nM (Cole et al., 2005b). It should be noted that *R*-13c showed affinity for other serotonin receptors, 5-HT_{1B} (K_i=15), 5-HT_{1D} (*Ki*=29, n=1), 5-HT_{1F} (*Ki*=16) and 5-HT_7 (K_i=74). The same year EMD 386088 (5-chloro-2-methyl-3-(1,2,3,6-tetrahydropyridin-4-yl)-1*H*-indole) was synthesised and found to be a full and potent agonist at the 5-HT_6

receptor, with an EC_{50}=1.0nM in a cAMP assay. EMD 386088 elicited high selectivity for the 5-HT_6 receptor (IC_{50} of 7.4nM) which was 20-fold greater than other serotonergic and dopaminergic receptors other than the 5-HT_3 receptor (IC_{50} of 34nM, (Mattsson et al., 2005)).

The pharmaceutical company, Esteve, have developed two 5-HT_6 receptor agonists, both of which are high affinity 3-aminoalkylindole sulfonamides, 6-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*indol-5-yl)imidazo[2,1-b]thiazole-5-sulfonamide (E-6801) and 5-chloro-*N*-(3-(2-(dimethylamino)ethyl)-1*H*-indol-5-yl)naphthalene-2-sulfonamide (E-6387, (Romero et al., 2006)). Both compounds induced cAMP formation at the rat 5-HT_6 receptor, with pK_i values of 8.46 and 9.13, and pEC_{50} values of 8.02 and 6.52 accordingly.

Cole and colleagues (2007) discovered a novel 5-HT_6 receptor agonist, N_1-(6-chloroimidazo[2,1-*b*][1,3]-thiazole-5-sulfonyl)tryptamine (11q), found be fully potent and have high affinity for the 5-HT_6 receptor, K_i=2nM, EC_{50}=6.5nM and E_{max}=95.5%. When tested against other receptors and ion channels 11q was found to be highly selective for the 5-HT_6 receptor with >50-fold selectivity elicited. Brain to plasma ratio following intraperitoneal (i.p.) and oral (p.o). administration indicated moderate penetration of the BBB. *In vivo* studies provided evidence for an increase in extracellular GABA concentrations following administration of 11q, these GABAergic effects were inhibited with 5-HT_6 receptor antagonist treatment (Cole et al., 2007).

Ligand Name (Agonists)	Affinity	Potency	Agonist properties	Additional information
EMDT [1]	K_i=16nM. Affinity for 5-HT_{1A} (K_i=170nM), 5-HT_{1D} (K_i=290nM) and 5-HT_7 (K_i=300nM)	ED_{50}=3.6	Activated AC	-
R-13c [2]	K_i=1nM 5-HT_{1B} (K_i=15), 5-HT_{1D} (Ki=29, n=1), 5-HT_{1F} (Ki=16) and 5-HT_7 (K_i=74)	EC_{50}=1.1nM	-	-
EMD 386088 [3]	-	EC_{50}=1nM	-	IC_{50}=7.4nM (5-HT_6) IC_{50}=34nM (5-HT_3)
E-6801 [4]	pK_i=8.46	pEC_{50}=8.02	Induced AC	
E-6387 [4]	pK_i=9.13	pEC_{50}=6.52	Induced AC	
N_1-(6-chloroimidazo[2,1-*b*][1,3]-thiazole-5-sulfonyl)tryptamine (11q) [5]	K_i=2nM	EC_{50}=6.5nM		E_{max}=95.5%
WAY-181187 [6]	2.2nM	EC_{50}=6.6 nM	-	E_{max}=93%
WAY-208466 [6]	4.8nM	EC_{50}=7.3nM	-	E_{max}=100%

Table 1-3- Overview of 5-HT_6 receptor agonists and their properties.

[1] Glennon et al., 2000. [2] Cole et al., 2005b. [3] Mattsson et al., 2005. [4] Romero et al., 2006. [5] Cole et al., 2007. [6] Schechter et al., 2008

WAY-181187 and WAY-208466, two novel and selective 5-HT_6 receptor agonists have been identified; both have high affinity for the receptor (2.2 and 4.8nM respectively) and act as full agonists. EC_{50}=6.6 and 7.3nM, and E_{max}=93 and 100% accordingly in cell lines. Both ligands were selective for 5-HT_6 receptors, with over 60-fold selectivity against serotonergic, dopaminergic and α_1-adrenoreceptors (Schechter et al., 2008).

A recent paper has documented a set of idenylsulfonamides as selective, potent 5-HT_6 receptor agonists (Alcalde et al., 2009). Structure-activity experimentation found that the nature of the indene scaffold, substitution of aminoethyl side chain and nature of the aryl(heteroaryl)sulfonyl group determined the optimal compound, *N*-(inden-5-yl)imidazothiazole-5-sulfonamide (compound 43, (Alcalde et al., 2009)). Radioligand binding studies found the indeylsulfonamides to have high affinities for the 5-HT_6 receptor, K_i values ≥20nM, compound 43 elicited a K_i of 4.5nM. Compounds were potent 5-HT_6 receptor agonists, with $E_{max} \geq 95\%$ and EC_{50} values 0.3-14nM (Alcalde et al., 2009).

1.3.5.8 Functional Roles of 5-HT_6 receptors

Prior to the development of selective 5-HT_6 receptor ligands, initial evidence of the functional role of 5-HT_6 receptors was provided by antisense oligonucleotides (AO) directed against the initiation codon region of the 5-HT_6 mRNA (Bourson et al., 1995; Sleight et al., 1996).

1.3.5.9 Behavioural syndrome

Initial studies found that intracerebroventricular (i.c.v) administration of 5-HT_6 AO in rats, which induced a 30% reduction in [^{125}I]-LSD binding sites, caused a behavioural syndrome of increased stretching and yawning. These behaviours were blocked with atropine, but not haloperidol, suggesting that the behavioural syndrome was not modulated by dopaminergic neurotransmission (Bourson et al., 1995). Following this, groups found treatment with 5-HT_6 receptor antagonists Ro 04-6790 and SB-271046 caused a similar increase in stretching behaviour, which was blocked with pre-treatment with scopolamine and atropine, but not methyl-atropine, providing evidence that the effect is centrally mediated (Bentley et al., 1999; Lindner et al., 2003; Sleight et al., 1998). Further support for cholinergic modulation via 5-HT_6 receptor blockade were findings that Ro 04-6790 had no effect alone on rotational behaviour in 6-hydroxydopamine (6-OHDA) lesioned rats but inhibited either scopolamine- or atropine-induced behaviours (Bourson et al., 1998). Other studies have failed to replicate these responses with 5-HT_6 AO and antagonists (Hamon et al., 1999; Yoshioka et al., 1998). These studies provided initial evidence that antagonism of the 5-HT_6 receptor causes a modulation of cholinergic neurotransmission.

1.3.5.10 Depression

Depression has been estimated to affect over 20% of the World's population, the most common treatment is with selective serotonin reuptake inhibitors (SSRIs), although potential therapies include drugs acting directly on specific 5-HT receptors, such as 5-HT_2 and 5-HT_6 (Schechter et al., 2005). Certain antidepressant drugs elicit high affinity for the 5-HT_6 receptor (Monsma et al.,

1993), supporting the role of ligands at this receptor in the treatment of depression. Genetic studies have analysed the effects of the C267T polymorphism on the 5-HT_6 receptor and the incidence of bipolar affective disorder and found no statistical links (Vogt et al., 2000). Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis is found in many patients suffering from depression (de Kloet et al., 2005), studies have illustrated an interaction between 5-HT_6 receptor and the HPA axis. 5-HT_6 receptor mRNA was increased within the hippocampus following an adrenalectomy, and treatment with SB-271046 increased glucocorticoid receptors in the hippocampus and reversed cognitive deficits induced through increased HPA axis activity supporting a role of these compounds on memory deficits in stress-related disorders (Marcos et al., 2008a; Yau et al., 1997).

The forced swim test and tail suspension task (commonly used preclinical test of antidepressants) show that the 5-HT_6 receptor antagonist SB-399885 significantly reduces immobility time consistent with having to anti-depressant-like effects in both rats and mice, similar to the tricyclic antidepressant reference compound, imipramine (Wesolowska and Nikiforuk, 2007). Contradictory findings were observed when testing the effects of SB-271046 in mice, SB-271046 had no effect on tail suspension when given alone and partially reversed the reduced immobility induced by the SSRI fluoxetine (Svenningsson et al., 2007). Furthermore, EMDT, a 5-HT_6 receptor agonist, reduced the immobility time in the tail suspension paradigm, which was prevented with the treatment of SB-271046 (Svenningsson et al., 2007).

Proteins which regulate synaptic plasticity within the brain, such as neurotrophic factors like brain-derived neurotrophic factor (BDNF), and Arc, are targets for the treatment of depression, a recent study found acute activation of 5-HT_6 receptor by agonist LY-586713 caused an elevation of hippocampal and cortical BDNF and Arc mRNA levels, which was attenuated with pre-treatment of SB-271046 (de Foubert et al., 2007). These findings further support a role of 5-HT_6 receptor ligands in the treatment of depression.

1.3.5.11 Anxiety

Contradictory reports have been found for the role of 5-HT_6 receptors in the treatment of anxiety. The conditioned fear stress task monitors freezing behaviour during testing periods as an index of 'anxiety' and is accompanied by increased prefrontal cortex (PFC) 5-HT release. Following i.c.v administration of 5-HT_6 receptor AO the increase of 5-HT was inhibited but no effect was observed upon the freezing behaviour or unconditioned rats (Yoshioka et al., 1998), suggesting a role for these receptors in anxiety. Other groups found anxiogenic responses to i.c.v administration of 5-HT_6 receptor AO in the social interaction task, decreased time spent interacting, and elevated plus maze, decreased time spent exploring the open arms (Hamon et al., 1999; Otano et al., 1999).

The 5-HT_6 receptor antagonist SB-399885 was tested on an array of pre-clinical paradigms to determine its anxiolytic effects. In the Vogel conflict test in rats SB-399885 caused an anxiolytic-like effect, similar to diazepam although not as pronounced (Wesolowska, 2008; Wesolowska and Nikiforuk, 2007). Lesions of 5-HT neurons induced by pre-treatment with *p*-chloroamphetamine, had no effect

on anxiolytic properties of SB-399885 in the Vogel conflict test (Wesolowska, 2008), suggesting this anti-anxiety behaviour of 5-HT_6 receptor blockade is independent of the integrity of 5-HT neurons.

Flumazenil (a benzodiazepine receptor antagonist) significantly reduced diazepam and SB-399885-induced anxiolytic effects, suggesting the anxiolytic property of SB-399885 may indirectly involve the benzodiazepine receptor or GABA modulation (Wesolowska, 2008). The four plate test was used on mice, SB-399885 significantly increased the number of punished crossings, similar to diazepam. These studies provide evidence of anxiolytic properties of SB-399885 in a variety of behavioural models in two different species (Wesolowska and Nikiforuk, 2007). One group analysed the effects of WAY-181187 on a model of obsessive compulsive disorder and found the rats treated with this agonist displayed an anxiolytic effect reducing the adjunctive drinking behaviour in the paradigm (Schechter et al., 2008). All these studies have indicated an interaction between GABAergic neurotransmission and 5-HT_6 receptors, which is supported by the neuroanatomical findings of 5-HT_6 mRNA and protein localised on GABAergic neurons (Gerard et al., 1996; Ward et al., 1995; Woolley et al., 2004).

1.3.5.12 Nociception

5-HT_6 receptors are located in areas of the brain that are associated with pain, indicating a potential role in nociceptive processing. Early studies observed an increase in the pain threshold of rats following administration of 5-HT into the amygdala (Plaznik et al., 1985). Following this, groups analysed the effects of ligands at the 5-HT_6 receptor on nociception, an initial study utilised the hot-plate

paradigm, this group found that administration of 5-HT_6 receptor AO had no effect upon reaction time (Bourson et al., 1995). Recent evidence has found that administration of the 5-HT_6 receptor antagonist SB-271046 caused a reduction in the formalin-evoked nociceptive behaviour elicited in rats (Finn et al., 2007). The difference in findings may be due to the different forms of pain as the hot-plate is a test of acute thermal pain whereas formalin causes a tonic persistent pain. Further work is required to elucidate the role of 5-HT_6 receptors on pain.

1.3.5.13 Seizure threshold

Various 5-HT_6 receptor antagonists have been tested in the maximal electroshock seizure threshold (MEST) test, it has been found that SB-271046, Ro 04-6790, SB-258510 (Routledge et al., 2000), SB-357134 (Stean et al., 2002) and SB-399885 (Hirst et al., 2006) all increased seizure threshold, with increases of 46-182%, however it should be noted that these increases are small in comparison to clinically established antiepileptic drugs like carbamezpine (Upton et al., 1998). Anti-convulsant activity correlated with the brain and plasma concentrations of the compounds, therefore Ro 04-6790 may have a smaller effect upon seizure threshold (46%) due to a poorer penetration into the CNS than other 5-HT_6 receptor antagonists. Routledge did not observe any overt sedative effects in rats treated with SB-271046, suggesting that the effect upon seizure threshold is a direct action and not secondary to a depressant action of the test compound (Routledge et al., 2000).

1.3.5.14 Feeding

Several studies have implicated the 5-HT_6 receptor in the regulation of food intake. Initial studies found that administration of 5-HT_6-directed AO for four days had no effect upon food intake or body weight in rats (Hamon et al., 1999; Otano et al., 1999). Further research found contrasting results to these initial findings, a reduction in both food consumption and body weight were observed following treatment with 5-HT_6-directed AO for six days, and further support of the role of the 5-HT_6 receptor in feeding was the fact that Ro 04-6790 caused a reduction in body weight (Woolley et al., 2001). The weight gain following cessation of 5-HT_6 receptor antagonist treatment was slow, and rats never fully compensated for the loss of weight during treatment (Woolley et al., 2001). Both body weight and food intake are reduced following treatment with the 5-HT_6 receptor antagonists BVT 5182 and PRX-07034 in normal and diet-induced obese rodents via enhancing satiety (Heal et al., 2008). A recent study has found significant reductions in body weight and food intake of rats during a 4 week treatment with the agonist E-6837 (Fisas et al., 2006). Mice carrying a mutant 5-HT_6 receptor gained less body weight and consumed less food during an 11 week testing period than control group (Frassetto et al., 2008). These findings support a role of the 5-HT_6 receptor in treating obesity, and evidence suggests ligands acting upon the receptor are a promising pharmacological treatment for obesity.

1.3.5.15 Schizophrenia

Due to the high affinity of several atypical antipsychotics for the 5-HT_6 receptor much early interest focussed on their potential role in schizophrenia (Roth et al., 1994; Roth et al., 2004). Genetic association studies have been performed to try

and elucidate if polymorphisms within the 5-HT_6 receptor are associated with the disease, but there are contradictory findings. Through a systematic approach Ohmori and colleagues identified a trinucleotide repeat polymorphism, $GCC_{2/3}$ between base pairs 1093 and 1085, but this did not increase the incidences of schizophrenia in Japanese subjects (Ohmori et al., 2001).

Post-mortem analysis on human brains found that the distribution of [^{125}I]-SB-258585 binding was similar between human and rats, with sites in striatum, cortex and hippocampus, the distribution of [^{125}I]-SB-258585 binding correlates with the expression of 5-HT_6 receptor mRNA. In a comparison study between schizophrenic and control subjects no differences in [^{125}I]-SB-258585 binding or 5-HT_6 receptor mRNA expression within the dorsolateral prefrontal cortex were observed (East et al., 2002a; East et al., 2002b). The same group found 5-HT_6 receptor mRNA was decreased within the hippocampal formation of schizophrenic patients when compared to age-matched controls (East et al., 2002a) however similar findings have been seen with many receptors and it is unclear whether these might be associated with antipsychotic drug treatment. Thus chronic administration of clozapine, but not haloperidol, significantly reduced hippocampal 5-HT_6 receptor density (Zhukovskaya and Neumaier, 2000) and 5-HT_6 mRNA levels (measured by *in situ* hybridization) while no change was observed in cortical regions (Frederick and Meador-Woodruff, 1999), suggesting an association between atypical antipsychotics and 5-HT_6 receptors. One group found no differences in binding densities of 5-HT_6 receptor mRNA in rats following two weeks administration of atypical or typical antipsychotics (East et al., 2002b), discrepancies between findings may be due to methodological approaches taken by each group.

Prepulse inhibition (PPI) of acoustic startle is a measure of sensorimotor gating, which is often impaired in schizophrenia. The selective 5-HT_6 receptor antagonist, Ro 4368554, had no effect on PPI either when administered alone or after a scopolamine-induced deficit, yet it reversed the deficit induced by the dopamine agonist, apomorphine (Mitchell and Neumaier, 2008). Other groups found that SB-271046 dose-dependently antagonised the effects of D-amphetamine on PPI with similar magnitude to that of clozapine (Pouzet et al., 2002). The same group found SB-271046 had no effect upon D-amphetamine induced hyperactivity and PCP deficits in PPI, suggesting the 5-HT_6 receptor antagonist does not elicit antipsychotic actions (Pouzet et al., 2002).

The reduced extra-pyramidal effects of atypical antipsychotics are not due to their affinity at 5-HT_6 receptors as 5-HT_6 receptor antagonists, Ro 04-6790 and SB-271046, did not induce catalepsy (Bourson et al., 1998; Pouzet et al., 2002), nor did Ro 04-6790 exert an effect upon haloperidol or SCH 23390-induced catalepsy (Bourson et al., 1998). Ro 04-6790 did not induce rotational behaviour in 6-OHDA lesioned rats, nor did it have an effect on L-DOPA or amphetamine-induced deficits (Bourson et al., 1998). These results suggest no modulation of the dopaminergic system via the 5-HT_6 receptor, although this is not supported by recent neurochemical data. Oral administration of SB-271046 was found to significantly increase extracellular levels of DA and NE in the mPFC measured by *in vivo* microdialysis, without altering 5-HT neurotransmission (Lacroix et al., 2004). This is different from findings by Dawson et al (2001), who reported a slight increase in DA within the frontal cortex but failed to reach significance, the

contradictory findings from the two studies may be due to the measurements being read from different regions of the cortex. The results from Lacroix et al (2004) show similar neurochemical measurements to those following atypical antipsychotic treatment, olanzapine has been shown to increase DA and NE in the mPFC (Li et al., 1998). The efficacy of atypical antipsychotics to treat cognitive symptoms of schizophrenia may be due to the observed changes in the monoamines, which could be elicited by their actions upon the 5-HT_6 receptors.

1.3.5.16 Cognition

Due to the distribution of 5-HT_6 receptors in several areas of the brain known to be involved in cognitive processes, much research has focussed on elucidating the effects of this receptor, and compounds that act upon it, on learning and memory (Mitchell and Neumaier, 2005; Russell and Dias, 2002; Terry et al., 2008). Recent work on patients suffering from AD found an imbalance in the serotonergic system and a reduction in the density of 5-HT_6 receptors within the frontal and temporal cortex (Garcia-Alloza et al., 2005; Garcia-Alloza et al., 2004), and although the findings from the paper highlighted the non-cognitive symptoms correlated with these findings it is known the cortical region of the brain plays a crucial role in cognition, and therefore this cannot be overlooked. Polymorphisms of the 5-HT_6 receptor have been associated with AD, positive correlations being documented between the C267T allele and AD in Chinese and Caucasian populations (Alvarez-Alvarez et al., 2003; Tsai et al., 2000) suggesting a possible therapeutic use of 5-HT_6 receptor ligands on AD.

As previously stated, initial studies performed had to use AO to determine the effects of 5-HT_6 receptors, Woolley and colleagues (2001) determined the effects of i.c.v administration of 5-HT_6 AO and i.p. injection of Ro 04-6790 on Morris water maze (MWM). Neither compound had an effect on acquisition, but both enhanced retention, such that AO-treated rats spent significantly longer searching the quadrant where the platform had previously been positioned compared to controls (Woolley et al., 2001). Many studies have confirmed these findings with other 5-HT_6 receptor antagonists. Results found sub-chronic or chronic administration of SB-271046, SB-357134 and SB-399885 in normal or aged-impaired rats enhanced spatial learning (Foley et al., 2004; Hirst et al., 2006; Marcos et al., 2008b; Rogers and Hagan, 2001; Stean et al., 2002), but no pro-cognitive effects were observed in healthy subjects treated with RO 4368554 (Schreiber et al., 2007). In age-induced, impaired rats, antagonism of 5-HT_6 receptors improved task acquisition and recall (Foley et al., 2004; Hirst et al., 2006). Marcos and colleagues (2008b) found that both cholinergic (scopolamine), and glutamatergic (MK-801), induced impairments of learning in MWM were reversed with co-administration of SB-271046 and galantamine.

Much research has focussed on the cognitive-enhancing properties of 5-HT_6 receptor compounds on cognitive deficits in schizophrenic patients, which includes tasks reliant on frontal lobe function. One test used to assess this is the Wisconsin card sorting task (WCST) in humans, which tests the ability to shift attention between stimuli presented, in rodents the attentional set-shift task is analogous to the WCST in humans. The test can involve altering a rule of discrimination within the same stimuli, intra-dimensional (ID) shift, changing the

attention to a different stimulus, extra-dimensional (ED) shift, or reversal learning. Two 5-HT_6 receptor antagonists, SB-399885 and SB-271046, administered sub-chronically for eight days were found to improve attentional set shifting in rats, with a reduction in the number of trials to meet the criterion on the test day, and for reversal and abolishment of ID/ED shift (Hatcher et al., 2005). A different group found that acute dosing with the agonist WAY 181887 facilitated the ED shift in the attention set shift paradigm, it was found to be dependent on 5-HT_6 receptors as administration of SB-399885 blocked the facilitation (Burnham et al., 2010).

Not all models of schizophrenia have found beneficial effects with 5-HT_6 receptor antagonist treatment, social interaction is a model of negative symptoms in schizophrenia, administration of phencyclidine (PCP, an NMDA receptor antagonist) caused significant disruption in the social interaction task which was not reversed with SB-271046 (Pouzet et al., 2002). Cortical hypofunction of glutamatergic neurotransmission is thought to be associated with schizophrenia, as NMDA receptor antagonists cause a range of pyschotomimetic effects.

One preclinical model utilised the NMDA receptor antagonist, MK-801, to induce hypermotility and ataxia in rats which was fully reversed with Ro 04-6790 (Pitsikas et al., 2008), suggesting a use for 5-HT_6 receptor antagonists in the treatment of schizophrenia. Although there are contradictory reports the findings suggest a role for 5-HT_6 receptor compounds in the cognitive deficits apparent in schizophrenia.

Novel object recognition (NOR) task measures recognition memory (thought to have translational relevance to visual learning and memory deficits seen in schizophrenia and AD) in rodents, to induce a deficit the inter-trial interval (ITI) can be lengthened when required. One group tested the effect of a 1 minute ITI, no effects were observed following treatment with Ro 04-6790 alone (Woolley et al., 2003). Cognitive deficits were induced using scopolamine and raclopride (a D_2 receptor antagonist), Ro 04-6790 reversed a scopolamine-, but not a raclopride-, induced memory deficit, supporting the theory that the blockade of 5-HT_6 receptors has a role on cholinergic neurotransmission (Woolley et al., 2003). One study found that Ro 04-6790 and SB-271046 reversed a 4 h ITI-induced deficit in NOR when administered 20 minutes prior to or immediately following the familiarisation trial, but not 20 minutes prior to the choice trial. Further analysis revealed this pro-cognitive effect upon recognition memory was modulated by glutamatergic neurotransmission as NMDA receptor antagonist, MK-801, prevented Ro 04-6790 from reversing the delay-induced deficit (King et al., 2004). Ro 4368554 had no cognitive enhancing effects on a time-induced deficit (24 hour) in a NOR paradigm, administration of metrifonate (AChEI) improved recognition memory. Cholinergic (scopolamine) and serotonergic (tryptophan (TRP) depletion) -induced memory deficits were reversed by administration of either metrifonate or Ro 4368554 (Lieben et al., 2005). Further analysis revealed the medium dose of Ro 4368554 only reversed the scopolamine-induced deficit suggesting cholinergic neurotransmission in NOR is more sensitive to the effects of Ro4368554. TRP depletion in the study by Lieben and colleagues (2005) implied the serotonergic system plays a role in the pro-cognitive effect observed with Ro 4368554, but this does not correlate with

the neurochemical data that found administration of 5-HT_6 receptor antagonists had no effect on 5-HT levels in striatum, dorsal hippocampus, nucleus accubmens or frontal cortex (Dawson et al., 2000, 2001). Other studies found RO 4368554 to reverse natural forgetting and scopolamine-induced memory deficit (Schreiber et al., 2007). Repeated administration for 7 days, of SB-399885 reversed a scopolamine-induced memory deficit in the NOR task, when given alone the 5-HT_6 receptor antagonist had no effect upon the retention test (Hirst et al., 2006). In a modified NOR paradigm, no effect upon memory was observed in young rats treated with scopolamine, BGC20-761 (5-methoxy-2-phenyl-*N,N*-dimethyltryptamine, PMDT, (Glennon et al., 2000)) or a combination of treatments, whereas mature rats elicited enhanced memory consolidation following treatment with BGC20-761 but no deficit was observed following scopolamine administration (Mitchell et al., 2006). This group used a 24 hour ITI which may have caused memory impairment in saline treated rats therefore in comparison scopolamine-treated rats did not differ from controls. Glutamatergic deficits, induced via administration of MK-801, in the NOR task were reversed with Ro 04-6790 treatment (Pitsikas et al., 2008), further supporting the proposal that the pro-cognitive effects of 5-HT_6 antagonism might involve enhanced modulation of glutamate neurotransmission.

Little literature is available regarding the effects of 5-HT_6 receptor compounds on social memory, and although social recognition is simpler in rats than humans, this task has particular relevance to schizophrenia. Systemic administration of WAY-181187, a 5-HT_6 receptor agonist, significantly impaired social recognition and this was attenuated with SB-271046 and SB-258585 treatment (Loiseau et

al., 2008). SB-271046, SB-258585, RO 4368554 and BGC20-761 reversed amnesia induced using scopolamine and an inter-session interval of 120 minutes in social recognition (Loiseau et al., 2008; Mitchell et al., 2006; Schreiber et al., 2007). Bi-lateral administration into the frontal cortex was of WAY-181187 was found to significantly impair social recognition, and SB-271046 administered into the frontal cortex, but not into the striatum or the NBM, significantly reversed the spontaneous deficit (Loiseau et al., 2008). These results further support the use of 5-HT_6 receptor compounds in treating cognitive dysfunction in CNS disorders.

5-HT_6 receptor antagonists, Ro 04-6790, SB-357134 and SB-399885, enhanced memory consolidation in naïve rats tested in an autoshaping task, irrespective of whether they were administered acutely or chronically (Meneses, 2001; Meneses et al., 2007; Perez-Garcia and Meneses, 2005). Further analysis revealed that this was associated with decreased 5-HT_6 receptor expression within areas of the brain, including hippocampus and amygdala (Meneses et al., 2007). Cholinergic and glutamatergic deficits were fully reversed with 5-HT_6 receptor antagonists, further supporting the modulatory role upon different neurotransmitter systems (Perez-Garcia and Meneses, 2005).

Fear conditioning is often used to test mnemonic properties of drugs. 5-HT_6 receptor compounds have little effect on their own on learning and memory processes in fear conditioning paradigms, although they have been found to reverse pharmacological induced deficits. Ro 04-6790 and SB-271046 reversed a scopolamine-induced deficit in the passive avoidance paradigm (Bos et al., 2001;

Foley et al., 2004) supporting a pro-cognitive effect of 5-HT_6 receptor antagonists in fear conditioning that relies upon the modulation of cholinergic neurotransmission. The fear potentiated startle paradigm is a form of fear conditioning, light and tone stimuli are paired with a foot shock, re-exposure to either the light or tone causes a startle response which is measured. Scopolamine prevented the potentiation of startle associated with the light, Ro 4368554 had no effect when administered alone, but reversed the scopolamine-induced deficit (Mitchell and Neumaier, 2008).

There are contradictory findings regarding the pro-cognitive effects of 5-HT_6 receptor antagonists; Lindner et al (2003) failed to replicate the mnemonic properties of Ro 04-6790 or SB-271046 in contextual fear conditioning (CFC), MWM or autoshaping tasks that other groups have found (Meneses, 2001; Meneses et al., 2007; Woolley et al., 2001). These discrepancies in results may be due to different drug administration times, or methodological and protocol differences (discussed in Chapter 3).

Neurochemical data support the findings from pre-clinical behavioural studies; *in vivo* microdialysis studies found SB-271046 had no effect on basal levels of 5-HT, DA or NE in either striatum, frontal cortex, nucleus accumbens or dorsal hippocampus, but produced a significant, tetrodotoxin (TTX)-dependent, increase in extracellular levels of both glutamate and aspartate in the frontal cortex and dorsal hippocampus (Dawson et al., 2000, 2001), which indicated the increase in excitatory neurotransmitter levels originated from glutamatergic neurons. Antagonism of cholinergic neurotransmission had no effect upon the increase in

excitatory neurotransmission observed in this study, indicating that the increase in excitatory neurotransmission was independent of the cholinergic system. These results were the initial studies to demonstrate enhancement of excitatory neurotransmission following antagonism of the 5-HT_6 receptor in areas of the brain involved in cognition. Results from these studies suggest that there is a tonic serotonergic inhibition of glutamatergic neurons which is modulated via 5-HT_6 receptors located on the GABAergic neurones (schematic of proposed model in Chapter 6).

SB-357134, SB-271046 and 4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine caused a concentration-dependent increase in K^+-evoked [^{3}H]ACh efflux in slices of striatum and cortex, both of which were blocked by tetrodotoxin (Marcos et al., 2006; Riemer et al., 2003; Shirazi-Southall et al., 2002). Administration of the glutamate release inhibitor, riluzole, into the frontal cortex, but not striatum, prevented the SB-357134-induced K^+-evoked [^{3}H] ACh release, suggesting the glutamate release within the cortex following administration of the 5-HT_6 receptor antagonist SB-357134 affects cholinergic neurotransmission (Marcos et al., 2006). Co-administration of SB-357134 (at the EC_{50} dose) and MK-801 enhanced K^+-evoked [^{3}H] ACh release, whereas given alone each compound was ineffective. SB-357134 significantly increased K^+-evoked glutamate release in frontal cortex and striatum. GABA levels were increased in striatal slices only, but no effect on SB-357134 induced [^{3}H] ACh release was observed following administration of $GABA_A$ receptor antagonist, bicuculline, therefore the GABAergic system is not directly affecting the 5-HT_6 receptor modulation of cholinergic neurotransmission. SB-357134 induced a

significant inhibition of DA release in striatal slices, these results contrast those of *in vivo* studies where SB-271046 enhanced DA release when followed by administration of amphetamine (Dawson et al., 2003; Marcos et al., 2006).

Within frontal cortex, striatum, amygdala and dorsal hippocampus, but not nucleus accumbens, WAY-181187 increased GABA concentration with no alteration of glutamate or NE. DA and 5-HT in the cortex were significantly reduced although the effect was modest. GABA and 5-HT alterations in cortex were blocked with pre-treatment of SB-271046, indicating these modifications were mediated through 5-HT_6 receptors. The decreased levels of DA and 5-HT were blocked with bicuculline, $GABA_A$ receptor antagonist, indicating a relationship between GABAergic neurotransmission and 5-HT_6 receptors. This effect was not ligand specific, as WAY-208466 administered acutely and chronically caused an increase in GABA levels, with a greater magnitude observed following chronic treatment (Schechter et al., 2008). In hippocampal slices 5-HT_6 receptor agonists attenuated the glutamate release following treatment with sodium azide and KCl.

Due to the evidence from behavioural and neurochemical pre-clinical studies utilising 5-HT_6 receptor ligands and the implications of serotonin in CNS disorders, 5-HT_6 receptor ligands are undergoing clinical trials (Table 1-4, (Heal et al., 2008; Upton et al., 2008)). One antagonist, SB-742457, has undergone two phase II clinical trials, showing efficacy in patients suffering from Alzheimer's (Upton et al., 2008). Further research is still required to determine the underlying effects of 5-HT_6 receptor compounds on learning and memory.

Compound	Phase	Status
SB-742457 (GSK)	Phase II	Treatment of AD, well tolerated in phase 1 trials
SAM-531 (Wyeth)	Phase II	Treatment of AD. Four phase I performed, phase II trial ongoing.
SGS-518 (Lundbeck/Lilly)	Phase II	Treatment of schizophrenia. Dose-dependent enhancement of cognition in small trial of schizophrenic patients
PRX-07034 (EPIX)	Phase I	Treatment of AD and obesity. Future phase II trial planned.
SYN-114 (Synosia/Roche)	Phase I	Treatment of AD.
SUVN-502 (Suven)	Phase I	Treatment of AD and obesity.
BVT-74316	Phase I	Treatment of obesity

Table 1-4- Overview of 5-HT_6 receptor ligands in clinical trials (for review see Heal et al 2008; Upton et al 2008).

1.4 Aims

As stated above there is substantial evidence from pre-clinical behavioural paradigms supporting a role of the 5-HT_6 receptor in learning and memory processes but the precise underlying mechanism is unclear. The aims of this thesis are:

- To perform a pharmacological characterisation and validation of a fear conditioning paradigm in rats.
- To elucidate the role of the 5-HT_6 receptor in a fear conditioning paradigm, by analysing the effects of antagonists and agonists on the learning and memory processes.
- To determine effects of 5-HT_6 receptor compounds on cholinergic- and glutamatergic-induced deficits in fear conditioning.
- To look at intracellular mechanisms involved in the actions of both 5-HT_6 receptor antagonists and agonists by examining changes in hippocampal protein expression following treatment in the CER paradigm.

1.5 Hypotheses

The fear conditioning paradigm will induce a robust memory in rats, which can be manipulated with drug treatment at various stages throughout the learning and memory process. The 5-HT_6 receptor antagonists will enhance memory in the CER task, reversing both a cholinergic and glutamatergic induced memory deficit. The 5-HT_6 receptor agonists will have no effect on memory in drug naïve

rats tested in the CER task, but will reverse a cholinergic and glutamatergic induced memory deficit.

2 Optimisation of a fear motivated learning paradigm in rodents: Conditioned Emotion Response

2.1 Introduction

Cognitive dysfunction is a common core symptom of many CNS disorders such as schizophrenia and AD. Schizophrenic patients are prone to suffer a deficit in working memory, attention and executive function (Lewis and Moghaddam, 2006), whereas patients with AD show a less specific overall, cognitive decline, with anterograde and retrograde amnesia (Nestor et al., 2006). Rodent cognitive behavioural tasks, such as the MWM and fear conditioning, have been used to study the normal process of learning and memory for many years. However, in recent studies these tasks have been used to observe cognitive dysfunction in animal models of CNS disorders, including AD and schizophrenia. Different stages of the learning and memory are affected by various CNS disorders, and the current chapter characterises and performs an initial pharmacological validation of a CER paradigm developed for use in rats at Nottingham, to permit associative memory acquisition, consolidation and retention to be investigated in subsequent chapters.

2.1.1 Learning and memory

A large literature exists on the underlying molecular and cellular mechanisms involved in the learning and memory process, but research into this area is still ongoing. Many pre-clinical studies have utilised fear conditioning/inhibitory avoidance (IA) paradigms to study the neurobiology of learning and memory because these paradigms utilise a single training session which reduces the confounding factor of repeated training upon the underlying intracellular pathways. Furthermore, fear conditioning in rodents elicit a strong response following a single training session indicative of robust memory. Findings from these studies indicate a biochemical cascade of events occurs in the hippocampus immediately following training (Figure 2-1), initially glutamate receptors are activated causing changes in second messenger systems and protein kinase (PK) A, C and G and calcium-calmodulin protein kinase II (CaMK II, (Frankland et al., 2004b; Izquierdo and Medina, 1997)). Studies have tried to identify the specific underlying pathways involved in the formation of such memory; Ahi and colleagues provided evidence that hippocampal PKA and PKC play a crucial role in fear conditioning, reducing the phosphorylation of Erk-1/2, Elk-1 and CREB. This group also found that inhibition of CaMKII did not affect fear conditioning (Ahi et al., 2004) which is contradictory to other groups (Mizuno and Giese, 2005), who have found that CaMKKα was required for contextual memory while interestingly CaMKKβ was required for spatial memory. ERK-1 deficient mice have elicited enhanced fear extinction, suggesting an up-regulation of MEK/ERK and down-regulation of ERK-independent PKC signalling is required for this particular form of learning and memory (Tronson et al., 2008). These enzymatic changes are followed by changes in glutamate receptors and an increase

expression of transcription factors (Frankland et al., 2004b; Izquierdo et al., 1997). As similar molecular processes are observed in the later stages of long-term potentiation (LTP) this has been proposed to be a candidate process for the consolidation of memory (Bear and Abraham, 1996; Bliss et al., 2006; Martin et al., 2000), as inhibition of LTP impairs hippocampal-dependent memories (Morris et al., 1986). LTP is a form of synaptic plasticity; increasing synaptic strength as a result of repetitive stimulation of interconnected neurons within cortical circuits (Bliss and Collingridge, 1993; Bliss and Lomo, 1973). In contrast, long-term depression (LTD) represents a decrease in synaptic strength, which is also believed to play a role in the storage of memories (Bear and Abraham, 1996). Considerable preclinical research has been performed to establish the cellular mechanisms underlying LTP and LTD in relation to learning and memory and evidence points to changes in hippocampal glutamatergic function (Malenka and Bear, 2004). Initial studies indicated that LTP and LTD were associated with phosphorylation and dephosphorylation of distinct sites of the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (Lee et al., 2000). Whitlock and colleagues then showed inhibitory avoidance training increased the phosphorylation of Ser 831 on the GluR1 subunit 30 minutes following the task, suggesting that LTP was induced during the early phase of learning and memory in the IA task (Whitlock et al., 2006). Research is ongoing into downstream molecular pathways underlying LTP and protein kinase M zeta (PKMζ) and activity-induced brain-derived neurotrophic factor (BDNF) have been identified as possible candidates (Lu et al., 2008; Pastalkova et al., 2006). Although there are many similarities between the early and late phases of LTP and short- and long-term memory (STM and

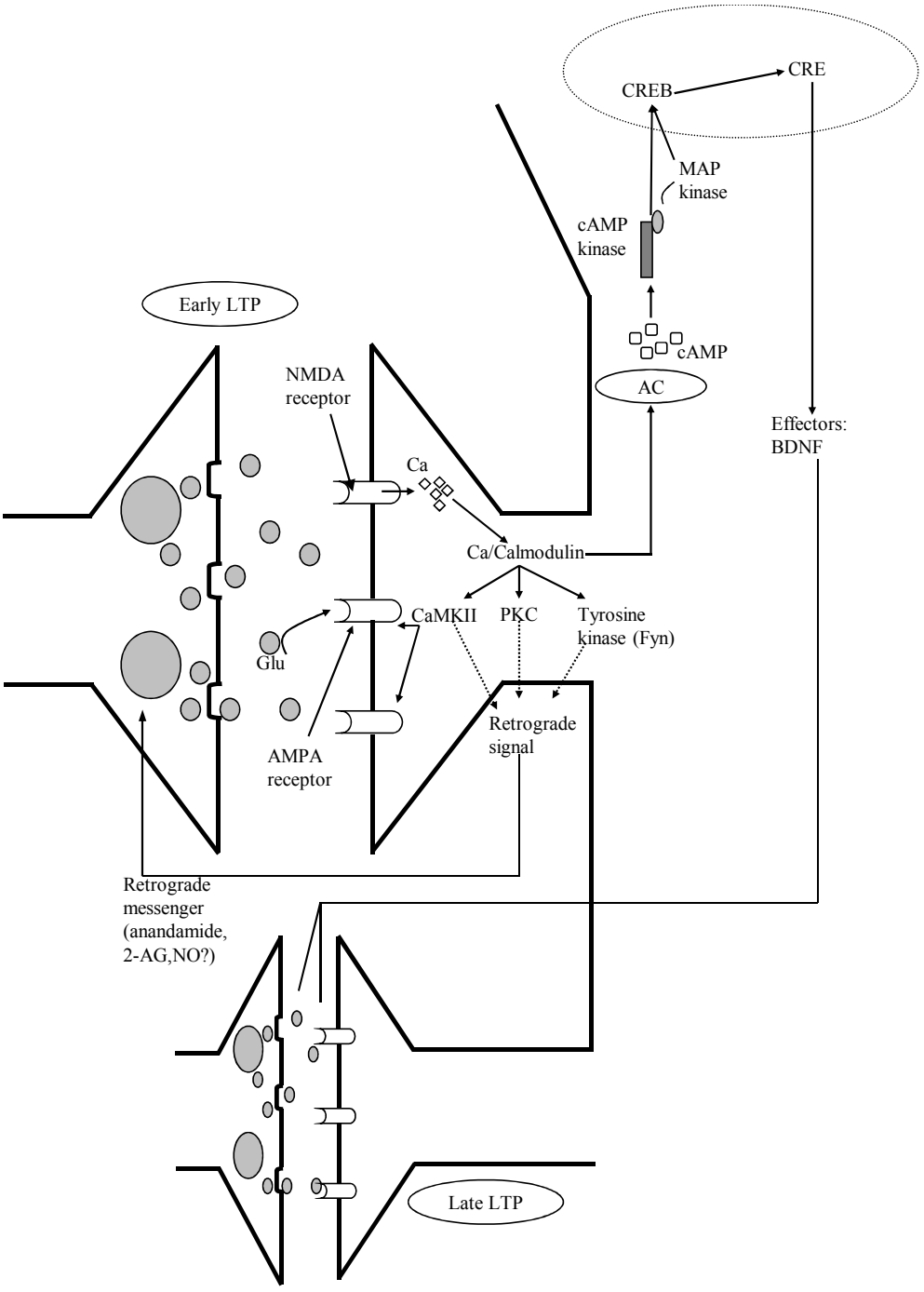


Figure 2-1- Schematic of signalling cascade following fear conditioning training. Ca = calcium, CRE = cAMP response elements, CREB = cAMP response elements binding protein, Glu = glutamate, LTP = long-term potentiation, MAP = mitogen activated protein, NO = nitric oxide.

LTM) respectively, it is not clear if LTP occurs in all forms of memory (Abraham and Williams, 2008). Many brain areas are involved in the process of learning and memory; one group illustrated the sequential role of the hippocampus, amygdala, entorhinal and parietal cortices in the IA task (Izquierdo et al., 1997). As stated in Chapter 1, it is known that the amygdala, in particular the basolateral amygdala (BLA) is required for the process of memory consolidation, retention and expression of extinction (Ehrlich et al., 2009; McGaugh, 2002). The process of memory retention has received less focus than acquisition and consolidation, but it is known that adrenergic neurotransmission is involved in the retrieval of contextual and spatial, but is not required for emotional memories (Murchison et al., 2004). Intra-amygdala injections of norepinephrine (NE) dose-dependently enhanced memory retention, via β-adrenoceptors, in a step-through IA task if administered immediately post-training. Furthermore the memory enhancing effects of peripheral epinephrine injection were blocked with the β-adrenoceptor antagonist, propranolol (Liang et al., 1986). In contrast to this epinephrine and amphetamine administered immediately post-training had no effect on memory in a classical fear conditioning paradigm (Lee et al., 2001), suggesting that different learning and memory mechanisms occur in the different fear conditioning tasks, or that the catecholaminergic drugs only effect memory in animals that can elicit both instrumental and classical conditioned responses. Fear extinction is enhanced in ERK-1 deficient mice, suggesting that extinction of fear requires up-regulation of MEK/ERK and down-regulation of ERK-independent PKC signalling (Tronson et al., 2008). It is clear that different stages of the learning and memory involve distinct brain regions and divergent neurotransmitter

systems, but further research is required to elucidate the specific candidates involved in the neuronal basis of learning and memory.

2.1.2 Conditioned Emotion Response

Conditioned emotion response (CER) is a form of Pavlovian classical conditioning. This form of associative learning was first described by Ivan Pavlov in the early 1900s, the original and famous experiment involving the salivation of dogs (Pavlov, 1927). CER training consists of pairing a conditional stimulus (CS), a context, light or tone, with an aversive unconditional stimulus (US), such as a foot shock. The CS is a neutral stimulus which if experienced alone or prior to CER would not be aversive to rats and, thus, would evoke no response on subsequent exposure to the context where it was received. In contrast, by pairing the CS with the US subsequently induces a conditioned response (CR) or learned behavioural response (freezing behaviour), which is only elicited on re-exposure to the context or cues used during the CS-US pairing. Freezing behaviour is a quantifiable response that can be used as an index of learning and memory (Blanchard and Blanchard, 1972; Blanchard and Blanchard, 1969). CER is commonly utilised to study learning and memory, as a single training session induces a reliable robust response without further CS-US pairings, making it an ideal model to investigate the underlying mechanisms of learning and memory without any confounding impact of training, handling or dietary manipulation.

2.1.3 Neuroanatomical and Neurochemical substrates of CER

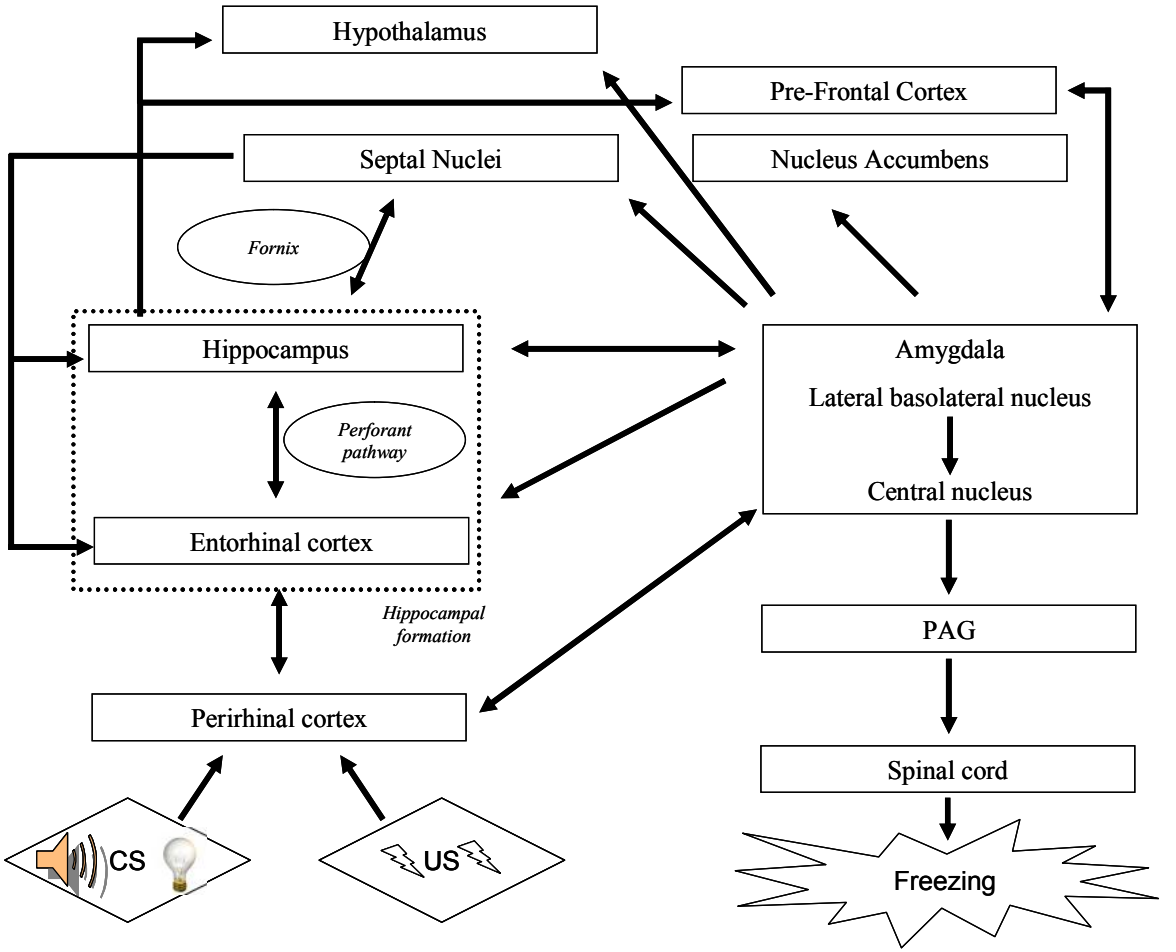
The strongest evidence for the neuroanatomical and neurochemical substrates involved in CER and other fear conditioning paradigms has been provided by brain lesioning and pharmacological manipulation. Lesions show the amygdala, midbrain periaqueductal gray (PAG) and hippocampus all play important roles in conditioned fear memories (Anagnostaras et al., 2001). Electrolytic lesions of the amygdala disrupt fear conditioning, impairing both contextual and cue-evoked fear responses from being observed in rats (Blanchard and Blanchard, 1972; Phillips and Ledoux, 1992). Furthermore, an infusion of the NMDA receptor antagonists, D,L-AP5, D-AP5, CPP, or APV, in the BLA prior to training causes a deficit in contextual fear and inhibitory avoidance in rodents (Kim and McGaugh, 1992; Maren et al., 1996), suggesting that activation of these receptors in the amygdala is required for the acquisition and consolidation of fear memories. Similar to this, Wilensky and colleagues found pre-training inactivation of the amygdala, via infusion of the $GABA_A$ receptor agonist muscimol, blocked auditory conditioning with no effect observed following post-training inactivation (Wilensky et al., 1999). These findings appear to contradict many studies analysing the effects of post-training blockade of the amygdala in IA tasks (Brioni et al., 1989; Izquierdo et al., 1997), but the apparent discrepancy may be due to the different types of conditioning used. Nonetheless, these studies show that the amygdala plays a crucial role in fear conditioning and associative learning independent of the CS presented during training.

Hippocampal lesions, made 1, 7, 14 or 28 days post-training, produce a deficit of contextual fear that is time-specific, suggesting that the hippocampus plays an

essential role in memory of CFC, but that this memory becomes independent of the hippocampus (Kim and Fanselow, 1992). This hypothesis was further supported by the same group (Anagnostaras et al., 1999a) who examined the temporally graded retrograde amnesia (RA) observed following hippocampal lesions in a within-subjects study design. Rats that received a dorsal hippocampal (DH) lesion 1 day post-training had severe deficits in that particular contextual fear, although they still showed fear of a paradigm they had been trained in 50 days prior to the lesion. NMDA-induced neurotoxic DH lesions made one week prior to contextual fear conditioning had no effect on behavioural acquisition, whereas post-training lesions, made 1 or 28 days post-training, induced an impairment of contextual fear. This was different to electrolytic lesion studies which caused a deficit in memory acquisition and retention (Maren et al., 1997). Electrolytic DH lesions may sever the projections between different brains areas as well as damaging the DH, this may account for the discrepancies between lesion types. It has been shown that the hippocampus plays a critical role in the contextual control of fear extinction in CER, and the amygdala is required to store and express this information. The medial prefrontal cortex (mPFC) plays a role in regulating the expression of fear extinction via inhibition of the amygdala (Bouton et al., 2006; Ji and Maren, 2007).

Kim et al (1993) analysed the effects of lesions on the amygdala, dorsal and ventral PAG (dPAG, vPAG), and the hippocampus on immediate and delayed freezing in a contextual fear paradigm. A significant attenuation of freezing behaviour elicited by rats immediately after shock administration was observed in subjects with lesions of the amygdala and vPAG, delayed freezing (24 hours

Figure 2-2- Schematic diagram of brain areas involved in conditioned emotion response. Lesioning studies have highlighted the hippocampus, amygdala and PAG as main areas involved in CER. Lesions to hippocampus disrupte contextual fear but cue-induced fear remained intact, whereas lesions to amygdala disrupted both contextual and cue-induced fear conditioning. CS = conditioned stimulus, US = unconditioned stimulus, PAG= periaqueductal grey.


Hypothalamus
Pre-Frontal Cortex
Septal Nuclei
Nucleus Accumbens
Fornix
Hippocampus
Amygdala
Lateral basolateral nucleus
Central nucleus
Perforant pathway
Entorhinal cortex
Hippocampal formation
PAG
Perirhinal cortex
Spinal cord
CS
US
Freezing

post-training) was attenuated by lesions of amygdala, vPAG and hippocampus. This study supports the theory of short- and long-term states of fear memory, and provides evidence for different neuroanatomical substrates being involved in these states (Kim et al., 1993). Thus, overall, there is clear evidence that multiple brain regions are involved in different aspects of learning and memory. For instance pre-training lesions of the hippocampal formation projecting to the BLA, or lesions in the BLA, caused significant impairments of Pavlovian conditioning (Maren and Fanselow, 1995) and lesions to the fornix prior to training, which is the primary pathway between the hippocampus and subcortical regions, also disrupts CFC, while no effects were observed in lesions to either entorhinal or entorhinal and perirhinal cortex (Phillips and Ledoux, 1995), which contradicts others (Izquierdo et al., 1997).

Pharmacological studies have shown that glutamatergic and cholinergic neurotransmission also play an important role in CER. Administration of the muscarinic receptor antagonist, scopolamine, or the non-competitive NMDA receptor antagonist, MK-801 (dizocilpine), produces deficits in both cued and contextual fear conditioning (Anagnostaras et al., 1999b; Csernansky et al., 2005). In addition, the attenuation in freezing behaviour observed following administration of these compounds has been reversed by pre-treatment with AChEI, such as physostigmine, donepezil and galantamine (Anagnostaras et al., 1999b; Csernansky et al., 2005; Lindner et al., 2006).

2.1.4 Aims

The aims of the experiments described in the current chapter were to validate and perform an initial pharmacological characterisation of a new conditioned emotion response paradigm in rats (developed at Nottingham University) that produces a pronounced freezing response, that can be quantified as a reliable index of learning and memory, on re-exposure to the context where the foot shock had been received, this was achieved by:

- Increasing number of CS-US pairings to determine the optimum number of foot shocks administered to evoke a sub-maximal freezing response that could be attenuated or enhanced by drug treatments.
- Testing the strength of fear-induced memories acquired in CER by analysing if fear extinction occurs upon re-exposure to the training context, and whether retention of memory still occurs with increased interval between training and retention trial.
- Analysing the effects of both contextual and cue CS presentations on memory in the retention trial.
- Altering the freezing response elicited via administration of pharmacological compounds known to enhance or attenuate learning and memory.

2.2 Materials and Methods

The experiments in the current chapter that validated and optimised the CER paradigm were performed by a colleague (Nola Clarke) and myself, this was to comply with Home Office guidelines.

2.2.1 Animals

Adult male Lister hooded rats (Charles River UK or University of Nottingham Biomedical Services Unit (BMSU), derived from Charles River stock) were used for all experiments performed throughout this thesis. Weights for rats are given for each experiment. Rats were allowed to acclimatise to BMSU for 1 week after purchased from Charles River UK. Rats were housed in groups of 3-5 and kept on a 12h light-dark cycle, lights on at 07:00h, with food (B & K, Universal small rodent chow, standard diet) and water *ad libitum*, no environmental enrichment was allowed other than a cardboard tube. Room temperature (21 ± 2°C) and relative humidity (55 ± 10%) were kept constant. Rats were handled for two consecutive days prior to behavioural testing. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, and approval of local ethical review committee.

2.2.2 Drugs

Scopolamine hydrobromide, methyl scopolamine and galantamine were purchased from Sigma-Aldrich (Dorset, UK). Donepezil was purchased from the US National Institute of Mental Health. All drugs were dissolved in sterile physiological saline (0.154M) and administered in volumes of 1 ml kg^{-1} intraperitoneally.

2.2.3 Apparatus

The CER apparatus used in the studies presented throughout this thesis utilised a modified shuttle box (PanLab, Figure 2-3) originally designed to test active and passive avoidance in rats but modified according to our instructions for use in the current CER experiments. The CER test arena comprised two equal square compartments (510 (W) x 250 (D) x 240 (H) mm internal; 580 x 360 x 305 mm external, PanLab, LE 916), one light and one dark chamber, separated by an automated computer-operated door (100 x 100 mm). A single speaker was built into the back wall, and each compartment had a separate light, both of which were connected to the shuttle box control unit (LE 900). Within each chamber there was an independent grid floor (19 stainless steel rods, 1 cm apart) linked to the shocker box (LE 100-26), and in turn the control unit. The position of the rat was detected in either chamber by individual weight transducers located below each grid floor, and was used to control the door closure and light, tone or foot shock delivery to the appropriate chamber. The control unit was operated by the software program, ShutAvoid v.1.8.2 (PanLab, Barcelona). Prior to each experiment the test arena was cleaned with 20% v/v ethanol to remove any olfactory cues. All experiments were performed in constant light (125 lux at floor level in the arena) between 8:00 and 16:00h, and video recorded.

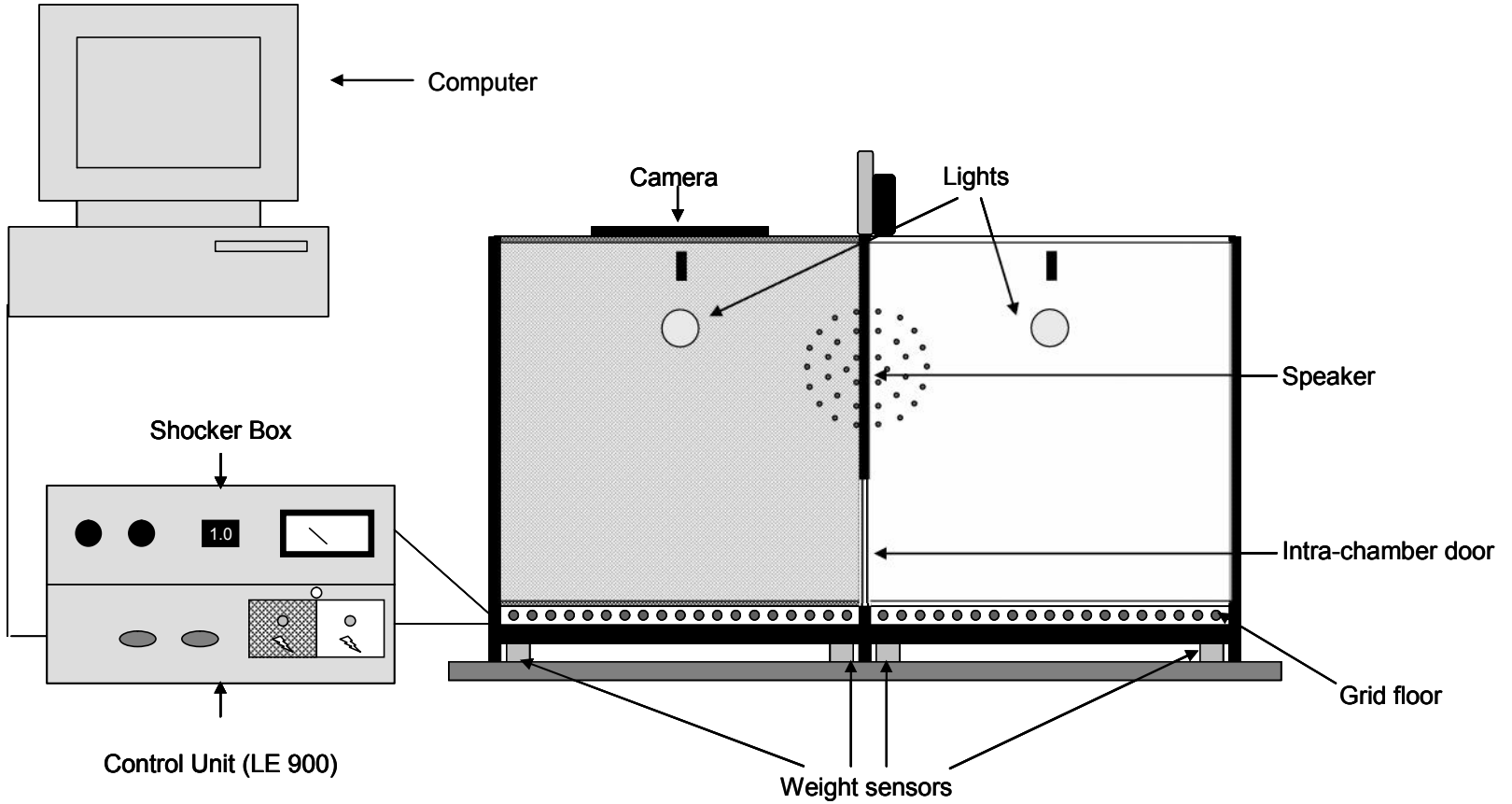


Figure 2-3-Schematic of CER apparatus utilised for all experiments.

2.2.4 Behavioural testing

2.2.4.1 Experiment I: Effects of increasing the CS-US pairings on CER-induced freezing in drug naïve rats

Rats, weighing 325-425g, n=7-8/group, were left to acclimatise in the behavioural suite, in a separate area to where CER training and testing was performed, for 1 hour prior to training. Individual rats were placed into the light chamber of the CER apparatus for 30 seconds free exploration then the intra-chamber door temporarily opened allowing transfer into the dark chamber. After a further 30 seconds of exploration the rat received 5 seconds combined light and tone (40 lux, 89 dB, 3kHz, CS), followed by an unavoidable foot shock (1 second, 0.4 mA, US). Following a 1 minute interval between each administration, the light, tone and shock were repeated to deliver a total of either one, three or five CS-US pairings. In these initial experiments each shock-treated group had a control group that received the CS with no US administration. Immediately following the fifth CS-US pairing, or at the equivalent time where fewer CS-US pairs were delivered, the rat was removed from the apparatus and returned to the home cage.

On the test day, 24 hours post-training, rats were left to acclimatise for at least 1 hour in a separate area of the behavioural suite. Individual rats were then placed directly into the dark chamber for 480 seconds; no stimuli were administered throughout the test trial. The total time spent freezing (defined as no movement other than respiration) was recorded manually using a stop watch by an observer blind to the treatment, who sat 1 metre from the apparatus, and test trials were

recorded by a camera located on the top of the dark chamber. Freezing behaviour is a quantifiable response that is used as an index of learning and memory (Blanchard and Blanchard, 1972; Blanchard and Blanchard, 1969; Bolles, 1970).

The CER protocol described above was used in all subsequent studies throughout this thesis with some modifications; three CS-US pairings were administered during training to allow for attenuation and enhancement of learning and memory to be studied, and the test trial was shortened to 300 seconds in the dark chamber.

2.2.4.2 Experiment II: Effect of extinction and varied time between training and testing on memory retention in drug naïve rats

Two separate groups of rats (n=6-8/experiment) were employed to determine the effect of extinction, and increased time between training and the retention test on memory in CER. To test the effects of extinction on memory retention drug naïve rats, weighing 365-455g, were re-exposed at 24, 48, 72 and 96 hours post-training to the dark chamber without receiving any further shocks. Freezing behaviour was recorded during each of the 300 second testing periods and would be expected to decrease as relearning occurred. To determine the effect of increased time between training and testing on memory in the CER paradigm without further exposure to the context, drug naïve rats, weighing 260-560g, were tested either 24, 48, 72 or 96 hours post-training.

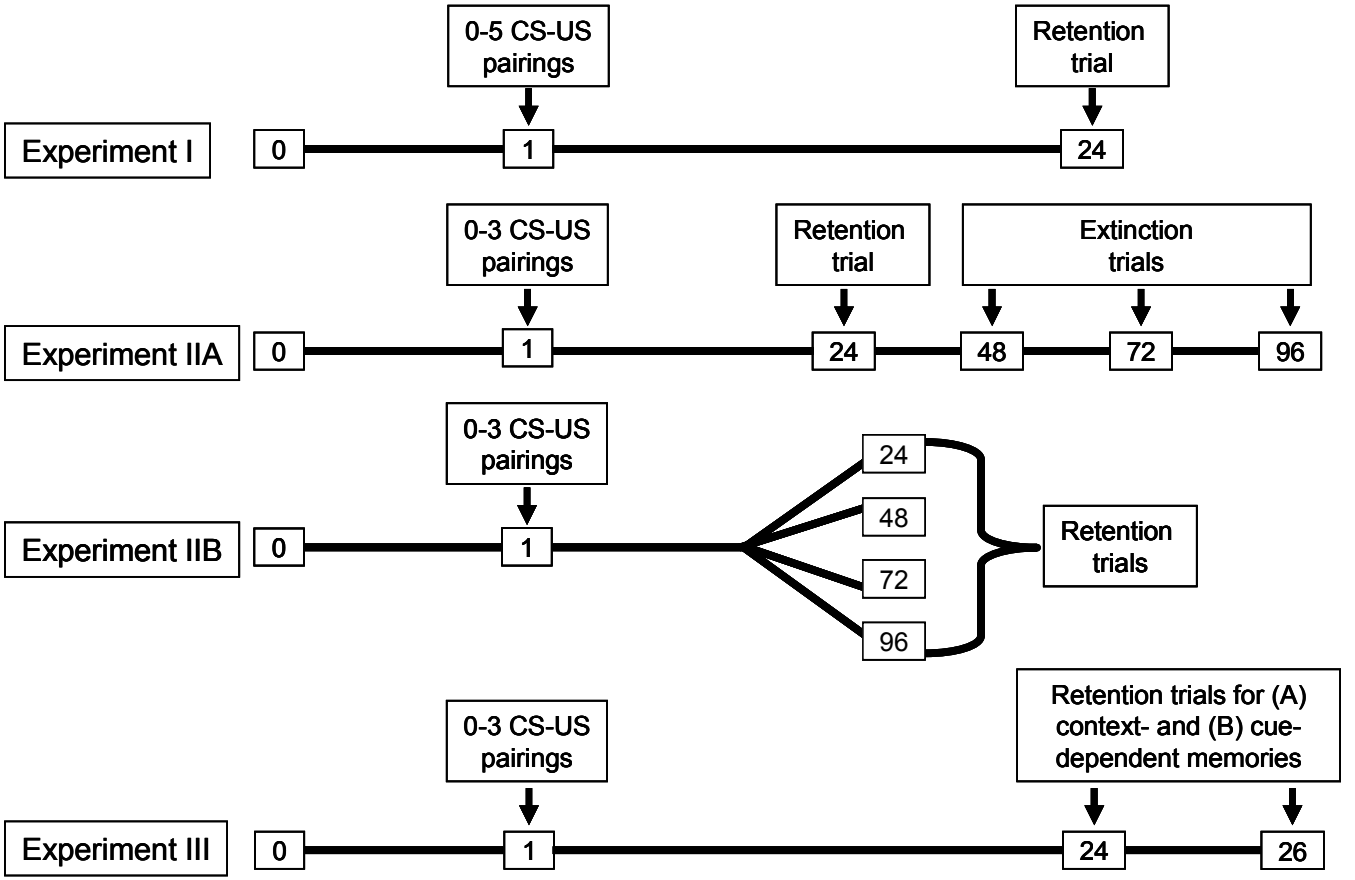


Figure 2-4- Schematic of experimental design for optimisation of CER in drug naïve rats. CS=conditioned stimulus, US=unconditioned stimulus

2.2.4.3 Experiment III: Effect of context and cue presentations on fear-motivated memory 24 hours post-training

The CER training protocol described above was performed on drug-naïve rats (n=6/group) to determine the extent of the association formed independently between both the context and cue with the shocks, 24 hours post-training. In this case on the test day rats (310-485g) were placed in the dark chamber and the context-associated memory was assessed as described above (Experiment I). To test the cue-associated memory a novel arena was used, which comprised a single metal and clear Perspex chamber (100 (W) x 90 (D) x 150 (H) external) with a built in speaker and light, and a grid floor (16 stainless steel rods, spaced 1 cm apart). Individual rats were placed in the novel chamber for 120 seconds prior to presenting the cue (5 seconds light and tone, 40 lux, 89dB, 3kHz), and a further 300 second test trial period recorded. In both trial periods the total time spent freezing was recorded as detailed before.

2.2.4.4 Experiment IV: Effects of pre-training administration of scopolamine and methyl scopolamine on CER-induced freezing

The CER protocol was performed as described in Experiment I using 3 CS-US pairings; memory retention was tested 24 hours post-training allowing for any cognitive effects of the muscarinic receptor antagonist, scopolamine, to be identified. To ensure that any effects observed during the task were due to the central effects of scopolamine rather than parasympatholytic effects on visual accuracy or other peripheral mechanisms, a separate group of rats received methyl scopolamine. As the methyl ester is charged at physiological pH and does

not cross the BBB (Anagnostaras et al., 1999b), this eliminated the possibility of peripheral autonomic effects impairing performance in the task. Rats, weighing 305-405g, were randomly assigned to one of four treatment groups (n=7-8); vehicle + no shock, vehicle + shock, methyl scopolamine shock or scopolamine + shock. Rats received a single injection of either scopolamine (0.3 mg kg^{-1}, i.p.), methyl scopolamine (0.3 mg kg^{-1}, i.p.) or saline (1 ml kg^{-1}, i.p.) 20 minutes prior to CER training, doses being selected from previous experiments (Anagnostaras et al., 1999b).

2.2.4.5 Experiment V: Effects of pre-training administration of donepezil and galantamine on CER-induced freezing

The CER protocol was performed as described in Experiment I; memory retention and extinction were tested 24-96 hours post-training, without further CS-US presentations, allowing for any cognitive effects of the AChEI, donepezil, or galantamine to be identified. Two separate groups of rats were assigned to test the effects of donepezil (n=6-8, weighing 250-305g) or galantamine (n=6-9, weighing 215-260g) on CER. Each experiment consisted of four groups; vehicle + no shock, vehicle + shock, donepezil or galantamine + no shock, and donepezil or galantamine + shock. Rats received a single injection of saline (1 ml kg^{-1}, i.p.) or donepezil (1.0 mg kg^{-1}, i.p.) 30 minutes prior to training, or saline (1 ml kg^{-1}, i.p.) or galantamine (3 mg kg^{-1}, i.p.) 40 minutes prior to training. Freezing behaviour was tested daily at 24, 48, 72 and 96 hours post-training.

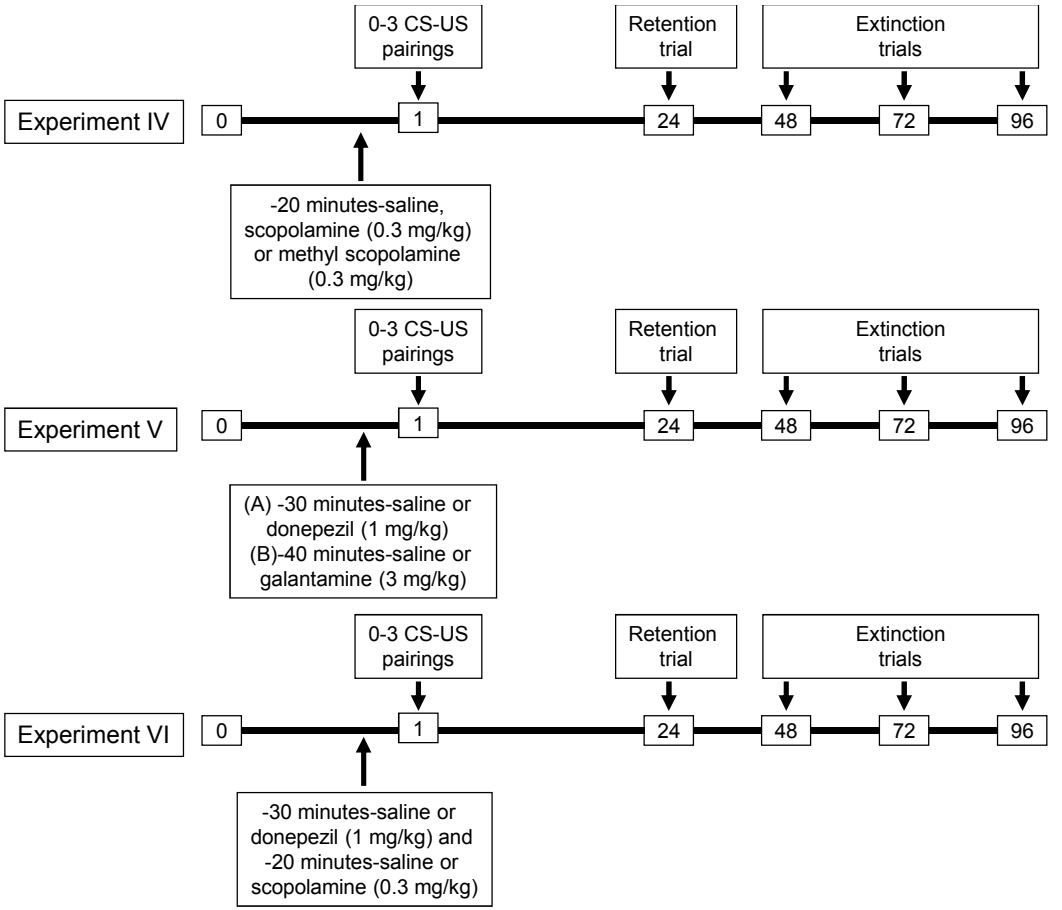


Figure 2-5-Schematic of experimental design for pharmacological manipulation of CER-induced freezing behaviour.

2.2.4.6 Experiment VI: Effects of donepezil on scopolamine-induced memory deficit

As an acute, pre-training injection of scopolamine (0.3 mg kg^{-1}, i.p., -20 minutes) caused an attenuation of freezing behaviour 24 hours post-training, this experiment examined the ability of donepezil to reverse this cholinergic deficit. Rats (n=6-9, weighing 280-340g) were randomly assigned to a treatment combination; saline + saline + no shock, saline + saline + shock, saline + scopolamine + shock, donepezil (1 mg kg^{-1}) + scopolamine + shock, and donepezil (2 mg kg^{-1}) + scopolamine + shock. As with experiment V, saline (1 ml kg^{-1}, i.p.) or donepezil (1 or 2 mg kg^{-1}, i.p.) was administered 30 minutes prior to training, and either saline (1 ml kg-1, i.p.) or scopolamine (0.3 mg kg^{-1}, i.p.) was administered 20 minutes prior to training as appropriate. Freezing behaviour was tested daily at 24, 48, 72 and 96 hours post-training.

2.2.5 Measured variables and statistical analysis

For all experiments the measured variables were latency to cross into dark chamber during CER training and total time spent freezing during testing trials. Statistical significance was achieved if $p \leq 0.05$, all data are illustrated as means ± s.e.m.

For latencies, Student's *t*-test or one-way ANOVA was utilised for all experiments. In experiment I a between-groups comparison of freezing behaviour was made using a two-way ANOVA (factors were shock treatment and frequency) followed by a Tukey's post-hoc test. Extinction trials were recorded

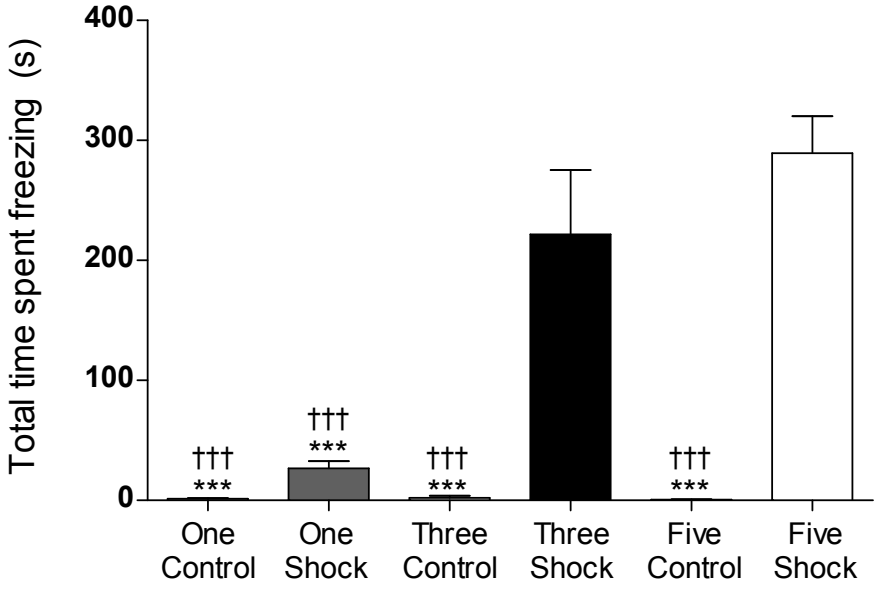
for experiments IIA, V and VI, therefore between-groups comparison of freezing behaviour were made using an ANOVA with repeated measures. Unpaired Student's *t*-tests were used to determine the difference between no shock and shock treated groups for experiments IIB (at each individual retention test), III and IV. For experiments III and IV a one-way ANOVA was also performed; for experiment III it was utilised to determine the difference in freezing behaviour during both pre- and post-cue administration, and in experiment IV it was used for between-groups comparison of freezing behaviour elicited between drug treatments.

2.3 Results

2.3.1 Experiment I: Effects of increasing the CS-US pairings on CER-induced freezing in drug naïve rats

No difference in latency times to cross into the dark chamber during CER training was observed between any treatment groups (data not shown). CER was optimised by analysing the effects of increased CS-US associations during training. Of particular importance rats that did not receive any shocks in the apparatus failed to elicit any notable freezing behaviour (<5 seconds) when re-exposed to the context 24 hours later. In contrast, increasing the number of foot shock pairings enhanced the memory (as measured by a very marked consistent freezing behaviour, two-way ANOVA of shock frequency and treatment $F_{(2,44)}$=12.317, p=0.001) 24 hour post-training, with the three and five shock-treated groups freezing for a significantly greater amount of time compared to the one shock group, and all the control groups ($p \leq 0.001$). Although no statistical

Figure 2-6- Increasing the number of CS-US pairings administered to drug naïve rats caused an increase in CER-induced freezing behaviour 24 hours post-training. Bar graph representing the total cumulative time spent freezing (seconds, mean ± s.e.m.) during 24 hour retention trial (n=7-8). *** $p<0.001$ versus three shock; ††† $p<0.001$ versus five shock (Tukey's post-hoc following two-way ANOVA).


Total time spent freezing (s)
400
300
200
100
0
†††
***
†††
***
†††
***
†††
***
One Control
One Shock
Three Control
Three Shock
Five Control
Five Shock

significance was observed in freezing between the three and five shock groups the latter caused a ~24% increase in freezing behaviour in rats compared to those given three CS-US pairings. Each shock group had its own control group, during the retention trial and each no-shock control group froze for less than 1% of the total testing period, indicating that rats did not find the CER testing apparatus sufficiently aversive to cause freezing, suggesting that anxiety to the environment did not have a major impact on the behavioural measures and further indicating that the freezing behaviour observed was a reliable index of fear conditioning 'memory'. As observed in the methods section, rats used in this experiment were varied in weight, it should be noted that rats were randomly assigned treatment and no differences were observed within treatment groups due to weight.

This data shows an increase in freezing behaviour with increased CS-US pairings, such that three CS-US pairings elicited a significant optimal freezing behaviour, three CS-US pairings were used for all subsequent studies.

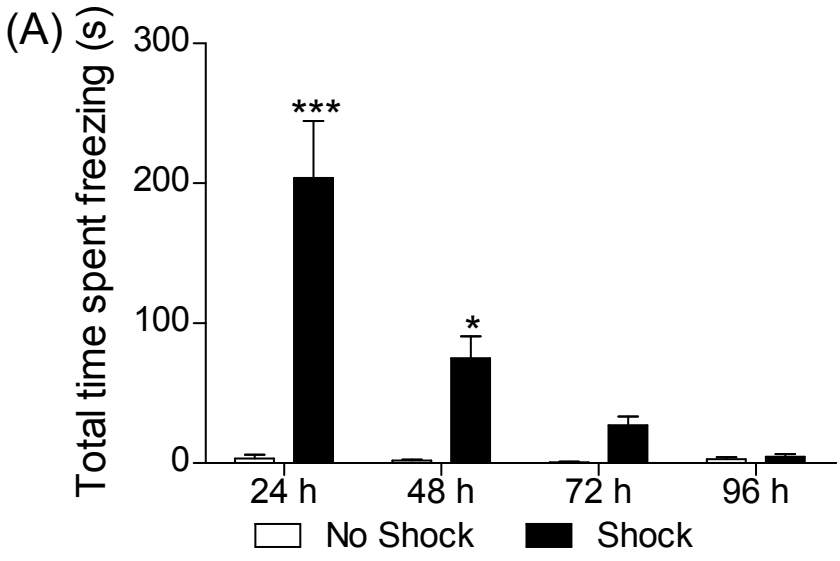
2.3.2 Experiment II: Effects of varied time between training and retention tests in drug naïve rats

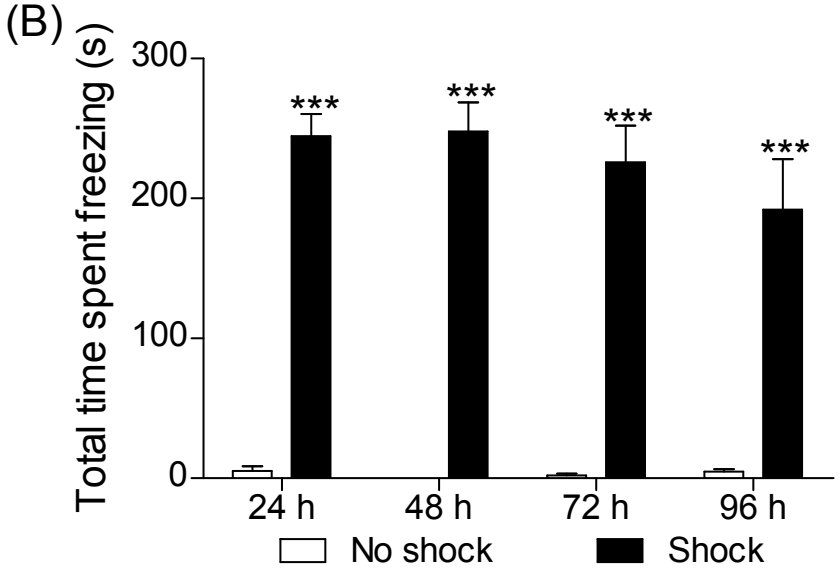
No statistical significance was observed between latency times to cross chambers during training (data not shown). Experiment IIA shows a clear extinction of the fear memory in a group of drug naïve rats that were tested at 24-96 hours post-training, repeated measures ANOVA showed a significant effect of shock $F_{(1,10)}=25.879$, $p=0.001$. Fear extinction is believed to occur when exposure to the CS (the context) is repeatedly provided without any further US association; it

has been shown that this process is not forgetting the previously learnt association but involves relearning that the context is no longer aversive (Ji and Maren, 2007). During the 24 hour retention trial shock treated rats froze for significantly longer than the no shock control treated group *($p \leq 0.001$)*, freezing for 67% of the total testing time. At 24 hour post-training trial the shock treated group froze significantly longer than all other groups and was longer than that seen on all subsequent extinction trials (Figure 2-7A), indicating a robust memory of the US during the retention trial. During the 48 hour trial, the shock treated group froze significantly more than the no shock treated group, but the amount of time spent freezing had decreased significantly compared to that seen in the 24 hours post-training session ($p \leq 0.001$). A progressive decrease in freezing behaviour was observed at the 72 and 96 hour time points as well, such that there was no longer any statistical significance between the shock and no shock treated groups at either of these extinction trial points.

Rats retained memory of the CS-US association up to 96 hours post-training if they have not been re-exposed to the context in the absence of a shock. Experiment IIB tested drug naïve rats, which received 0 or 3 shocks during training, once at various retention trials 24, 48, 72 and 96 hour post-training. No difference in latencies to cross into the dark environment between groups were observed (data not shown), this was expected as both groups had received identical behavioural protocols at that point. During each of the individual retention trials the rats that received shock treatment froze significantly longer than the no shock control group for that particular trial ($p \leq 0.001$, Figure 2-7B), confirming the strong association of the context with the aversive stimuli.

Figure 2-7- CER induced a robust memory that (A) underwent extinction when rats were re-exposed to the training context at 24, 48, 72 and 96 hours post-training with no further CS-US pairings, and (B) was retained even with increasing the time between training and initial retention trial. Bar graph representing the total cumulative time spent freezing (seconds, mean ± s.e.m, n=6-8, no-shock treatment group represented by white columns, shock treatment group represented by black columns). *** $p \leq 0.001$; * $p \leq 0.05$ versus own no shock control group at that particular testing trial (Tukey's post-hoc following ANOVA, experiment IIA, and Student's one-tailed *t*-test, experiment IIB). No statistical differences were found between shock-treated groups at each retention trial (one-way ANOVA, $F_{(3,29)}=1.024$, $p=0.398$).


(A)
Total time spent freezing (s)
300
200
100
0
***
*
24 h
48 h
72 h
96 h
No Shock
Shock


(B)
Total time spent freezing (s)
300
200
100
0
***
***
***
***
24 h
48 h
72 h
96 h
No shock
Shock

Irrespective of the time between training and the following contextual test all rats that received shock treatment spent an equal amount of time freezing, such that between-groups ANOVA found no statistical difference in freezing time. All rats which received shock treatment froze over 60% of the total test time. Of note with this experiment was the large weight range of rats used, no differences in freezing behaviour due to weight was observed within-groups.

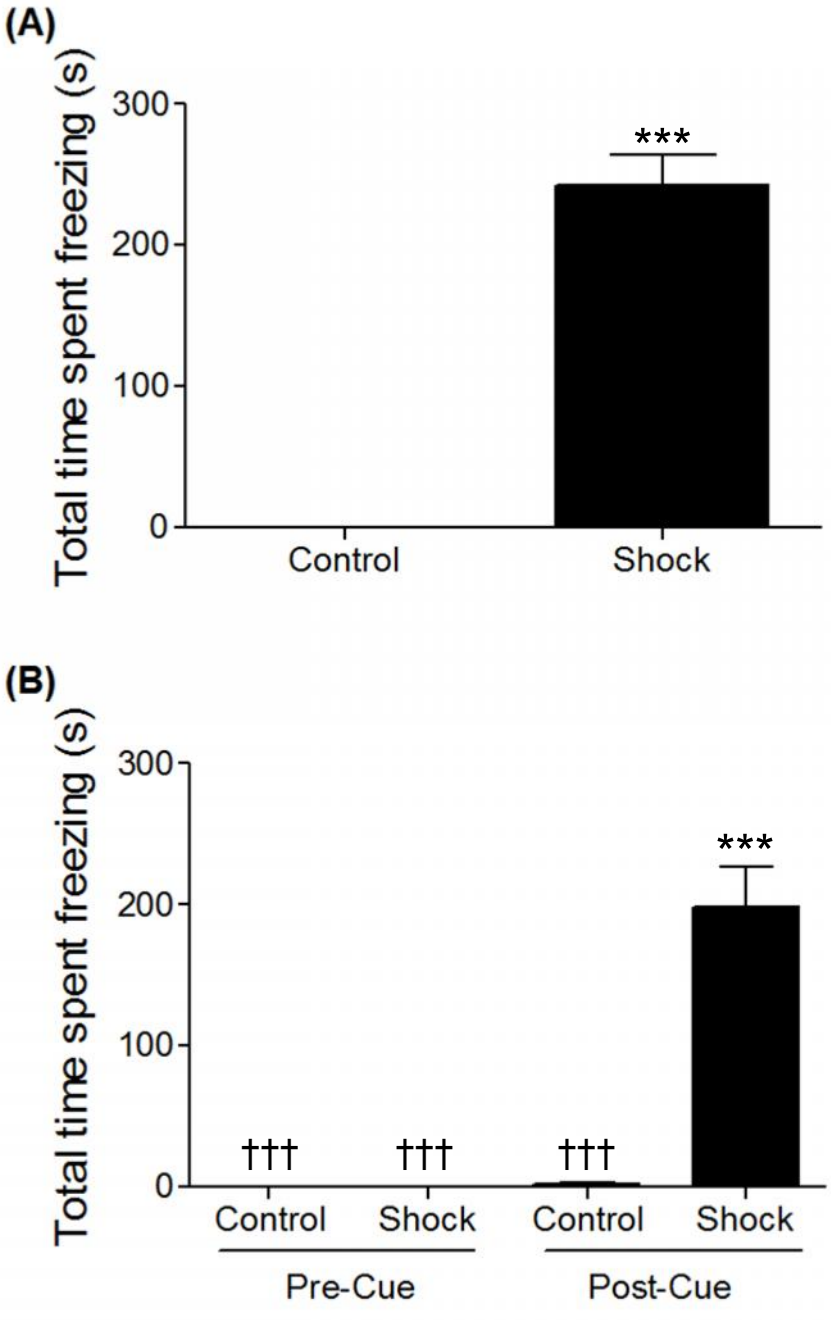
As with experiment I, no differences in freezing behaviour recorded were observed within treatment groups due to the weight range of the rats used in this experiment.

Combining the findings from this experiment it is clear that rats will learn that an environment is no longer aversive if re-exposed without further CS-US associations, but if no re-exposure occurs then a robust memory of the CS-US training context is retained for at least 96 hours post-training.

2.3.3 Experiment III: Effect of context and cue presentations on fear-motivated memory 24 hours post-training

Rats froze in response to both forms of conditioning stimuli that had been presented to them during CER training. No difference in latency time to cross into dark chamber was observed during training (data not shown). There was a large weight range of rats used in this experiment, analysis revealed no difference in freezing behaviour was observed (data not shown). Analogous to previous studies shock-treated rats froze for longer than 200 seconds, when tested 24 hours

Figure 2-8- Drug naïve rats elicited high levels of freezing behaviour (total cumulative time spent freezing (seconds, mean ± s.e.m), 24 hours post-training, when tested in both (A) original training context and (B) a novel context with cue administrations. *** $p \leq 0.001$ versus no shock control group; ††† $p \leq 0.001$ versus post-cue shock group (Student's *t*-test and Tukey's post-hoc following ANOVA, $F_{(3,23)}=46.019$, p=0.001).


(A)
Total time spent freezing (s)
300
200
100
0
***
Control
Shock
(B)
Total time spent freezing (s)
300
200
100
0
***
†††
†††
†††
Control
Shock
Control
Shock
Pre-Cue
Post-Cue

post-training in the context they received the CS-US pairing, which was a significantly greater amount of time than that of the no-shock treated rats (*p≤0.001*, Figure2-8A).

When placed in a novel arena (different from that used for administering CS-US pairings) all rats, irrespective of whether they had received a shock or not, elicited exploratory behaviour and did not spend any time freezing during the 120 second pre-cue test period. Following exposure to an identical cue to that presented during training, in the absence of any more shocks, rats that had received CS-US treatment froze (198.0 ± 29.1 s) for a significantly greater amount of time than to the no-shock-treated rats (1.7 ± 1.2 s, *p≤0.001*, Figure 2-8B).

This experiment illustrates that rats associate both the context and the cue presented to them during training with the foot shock in an independent manner, and when either of these modalities involved in the initial training are re-administered separately, freezing behaviour is elicited, indicative of fear conditioning.

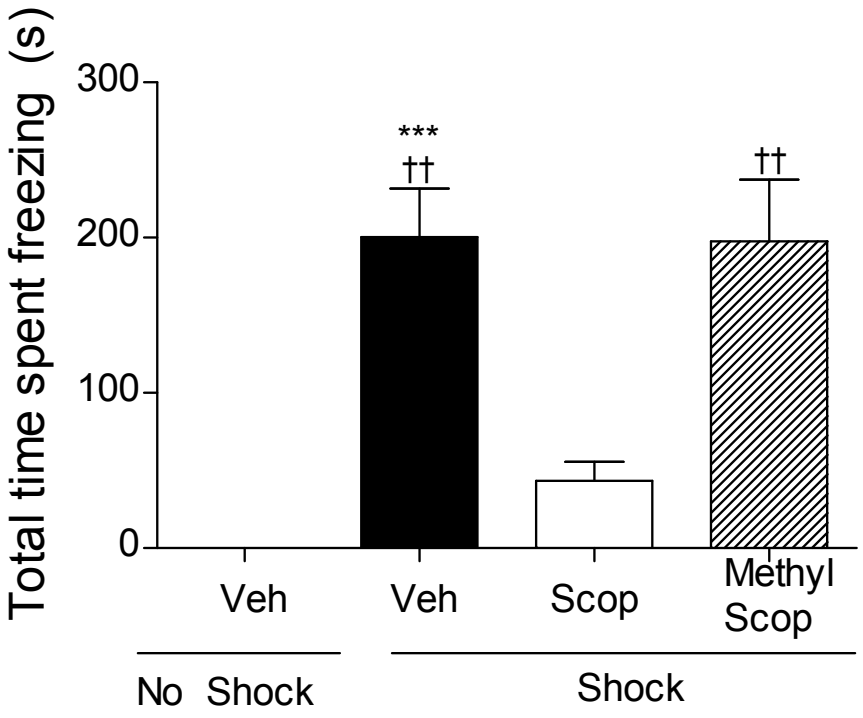
2.3.4 Experiment IV: Effects of pre-training administration of scopolamine and methyl scopolamine on CER-induced freezing

Pre-training administration of the muscarinic receptor antagonist, scopolamine, attenuated CER-induced freezing behaviour 24 hours post-training. Similar to

previous studies saline-treated shocked rats froze for a significantly greater time (200.5 ± 31.2 s) than the no-shock control-treated group (0.5 ± 0.5 s, $p \leq 0.001$, Figure 2-9). No statistical difference was observed between-groups for the time taken to cross into the dark chamber during CER training (data not shown), indicative that drug treatment was having no unwanted effects on rats. As both these groups required a pre-training saline injection, this experiment shows that this procedure had no adverse effect upon CER-induced behaviour. No differences were observed within treatment groups in freezing behaviour elicited due to weight range.

Between-conditions ANOVA ($F_{(2,22)}$=7.907, p=0.003) revealed a significant effect of treatment on CER-induced freezing behaviour. Treatment with scopolamine 20 minutes prior to training significantly reduced freezing behaviour in the 24 hour retention trial compared to that elicited by the shocked vehicle-treated group ($p \leq 0.01$). In contrast, pre-treatment with methyl scopolamine did not have any effect on CER-induced freezing behaviour, such that these rats spent a similar amount of time freezing to the vehicle-shock treated group (Figure 2-9). Scopolamine-treated rats elicited an attenuation of freezing behaviour when compared to the rats treated with methyl scopolamine ($p \leq 0.01$), supporting the role of central cholinergic pathways in mediating CER.

Figure 2-9- Scopolamine caused an impairment of CER-induced freezing behaviour (seconds, mean ± s.e.m.) 24 hours post-training. Rats (n=7-8/group) were pre-treated with saline (1 ml kg^{-1}), scopolamine (0.3 mg kg^{-1}), or methyl scopolamine (0.3 mg kg^{-1}). *** $p \leq 0.001$ versus no-shock control group (Student's *t*-test); †† $p \leq 0.01$ versus scopolamine-treated shocked group (Tukey's post-hoc following ANOVA $F_{(2,22)}=7.907$, $p=0.003$).


Total time spent freezing (s)
300
200
100
0
***
††
††
Veh
Veh
Scop
Methyl Scop
No Shock
Shock

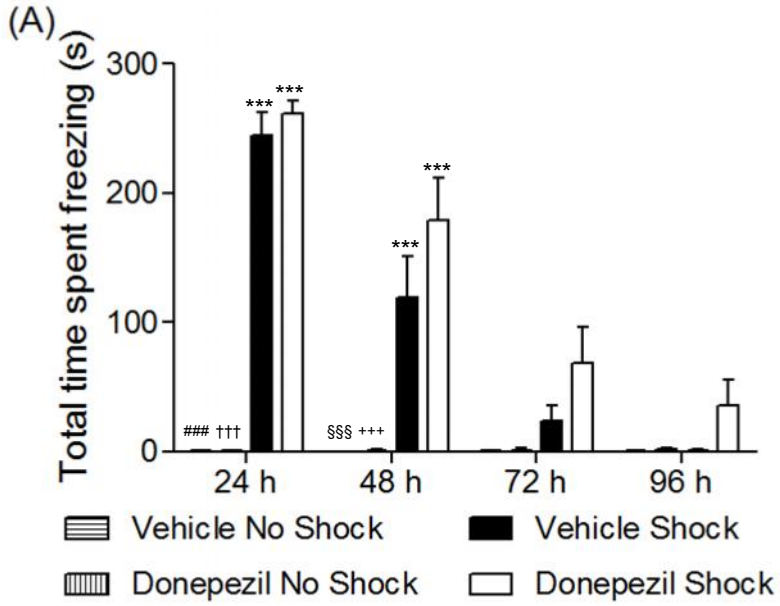
2.3.5 Experiment V: Effects of pre-training administration of donepezil and galantamine on CER-induced freezing

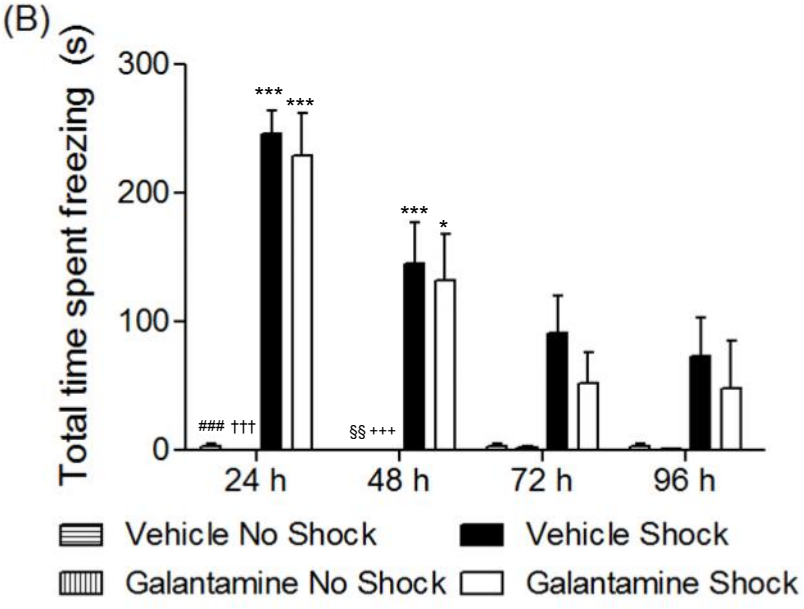
Pre-training administration of either donepezil or galantamine had no effect on CER-induced freezing behaviour during any of the testing trials. Neither experiment found a statistical difference in latency time to cross into the dark chamber during the conditioning trial, illustrating that the AChEI were having no overt motor effects on the rats.

Repeated measures ANOVA found a significant time shock interaction, but no effect of drug treatment. Consistent with the previous studies, vehicle-shock-treated rats, in both the donepezil and galantamine studies, had formed a strong association between the CS and US, and elicited significantly longer freezing behaviour 24 hours post-training than the saline no-shock-treated rats (Figure 2-10A and B). Both individual saline shock-treated groups showed clear fear extinction, with a reduction in total time spent freezing with each subsequent daily contextual test performed.

Donepezil and galantamine were administered to rats that did not receive a foot shock during training to test if these drugs had any non-specific effect that could mask the freezing behaviour. Neither donepezil nor galantamine altered freezing behaviour in the non-shocked rats compared to the saline no-shock-treated rats, illustrating that these AChEI had no confounding effect on the CER-induced freezing behaviour.

Figure 2-10- Neither of the acetylcholinesterase inhibitors (A) donepezil (1 mg kg^{-1}) or (B) galantamine (3 mg kg^{-1}) had any effect on CER-induced freezing behaviour (seconds, mean ± s.e.m). Rats received (A) saline or donepezil (n=6-8) prior to training or (B) saline or galantamine (n=6-9) prior to training. *** $p \leq 0.001$; * $p \leq 0.05$ versus own no-shock control group during the specific test trial, ††† $p \leq 0.001$ versus 24 hour saline shock group, ### $p \leq 0.001$ versus 24 hour donepezil or galantamine shock group accordingly, +++ $p \leq 0.001$ versus 48 hour saline shock group, §§§ $p \leq 0.001$; §§ $p \leq 0.01$ versus 48 hour donepezil or galantamine shock group (Tukey's post-hoc following repeated measures ANOVA).


(A)
Total time spent freezing (s)
300
200
100
0
***
***
***
***
†††
§§§ +++
24 h
48 h
72 h
96 h
Vehicle No Shock
Vehicle Shock
Donepezil No Shock
Donepezil Shock


(B)
Total time spent freezing (s)
300
200
100
0
***
***
***
*
†††
§§ +++
24 h
48 h
72 h
96 h
Vehicle No Shock
Vehicle Shock
Galantamine No Shock
Galantamine Shock

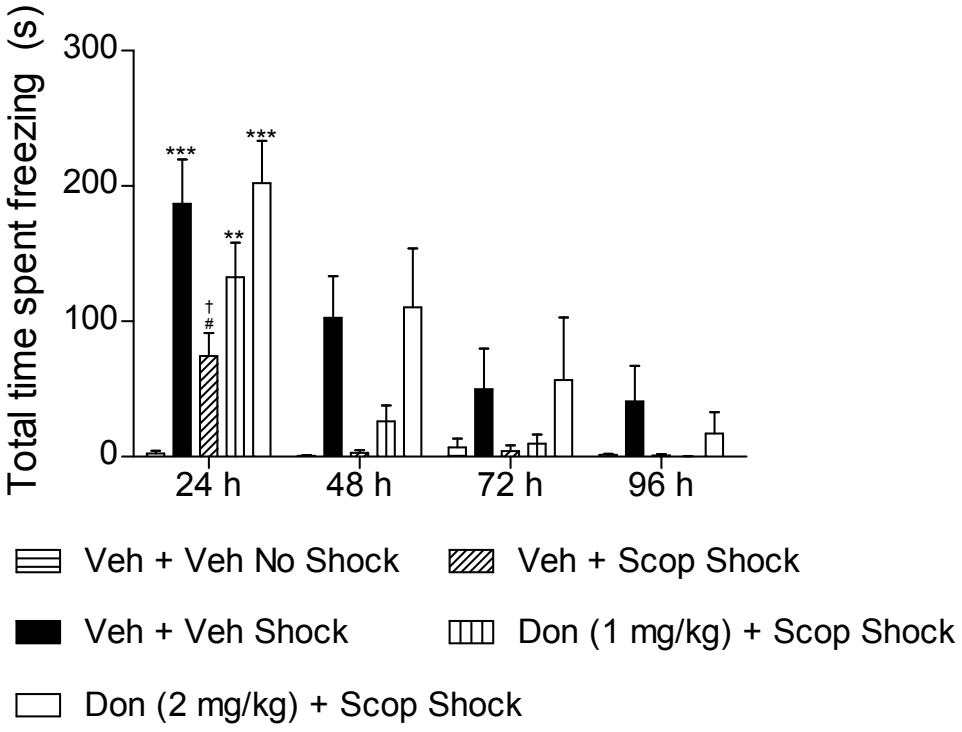
Repeated measures ANOVA did not show a statistical significance between shock and treatment over the four test periods ($F_{(1,25)}$=2.064, p=0.163). Although no statistical significance was observed between donepezil and saline shock-treated groups, donepezil treated rats froze slightly longer than saline controls at each post-training test trial, although the difference was more apparent at the later extinction trials. At ninety-six hours post-training donepezil shock-treated rats were the only group to freeze for a notable amount of time (35.4 ± 20.0 s, Figure 2-10A), suggesting that donepezil may have a pro-cognitive effect in rats trained at CER as the extinction process is taking longer to accomplish in these rats.

Galantamine had no effect upon the freezing response compared to saline control-treated groups (Figure 2-10**Figure 2-10**B). Extinction of the CER-induced memory occurred in all shock-exposed groups regardless of whether they had been treated with saline or galantamine. A greater amount of freezing behaviour was observed in saline shock-treated rats at later extinction points compared to that observed in other studies, this was due to one individual rat freezing for a substantial amount of time increasing the overall average of time spent freezing, this was not an outlier therefore was left in the experimental data.

2.3.6 Experiment VI: Effects of donepezil on scopolamine-induced memory deficit

This study examined the ability of donepezil to reverse a scopolamine-induced memory deficit 24 hours post-training. This experiment required two injections prior to training, -30 and -20 minutes but once again this protocol did not have

Figure 2-11- Donepezil (3 mg kg^{-1}) reversed the scopolamine-induced deficit on CER freezing behaviour (seconds, mean ± s.e.m). Rats (n=6-9) received donepezil or saline (-30 minutes), and saline or scopolamine (-20 minutes) prior to training. *** $p \leq 0.001$; ** $p \leq 0.05$ versus saline no-shock control group, † $p \leq 0.05$ versus 24 hour saline shocked group, # $p \leq 0.001$ versus 24 hour donepezil 2mg kg^{-1} (Tukey's post-hoc following repeated measures ANOVA, main effect of treatment $F_{(4,32)}=5.102$, $p=0.003$).


Total time spent freezing (s)
300
200
100
0
***
***
**
†
#
24 h
48 h
72 h
96 h
Veh + Veh No Shock
Veh + Scop Shock
Veh + Veh Shock
Don (1 mg/kg) + Scop Shock
Don (2 mg/kg) + Scop Shock

any adverse effects upon the behaviours elicited during the test trials such that freezing behaviour was comparable to that reported in previous experiments in this chapter.

Supporting this, no statistical significance was observed between-groups with time taken to cross into the dark chamber during training. Analogous with previous experiments saline-treated rats, that received a shock, had formed an association between the CS-US and spent a significant time freezing (186.8 ± 32.9 s) compared to the non-shock control group (2.4 ± 2.0 s) at the 24 hour retention trial ($p \leq 0.001$). Clear fear extinction was observed in the saline shock-treated rats with a reduction in the freezing behaviour at each subsequent test trial.

Scopolamine, administered 20 minutes prior to training, attenuated freezing behaviour by 60% of that seen in the saline shock-treated rats in the 24 hour retention test ($p \leq 0.05$, Figure 2-11

Figure 2-11), this is similar to the experiment IV in this chapter.

As donepezil had no effects upon freezing behaviour (experiment V) it was only administered to shock-treated rats in this experiment. Two doses of donepezil were administered to analyse the effects on scopolamine-induced deficits. At 1

mg kg^{-1}, donepezil failed to reverse the attenuation of freezing behaviour induced from pre-treatment with scopolamine ($p \geq 0.05$), with only a 44% increase in freezing behaviour (132.7 ± 25.3 s) compared to that of saline + scopolamine treated rats (74.5 ± 17.0 s). Donepezil (2 mg kg^{-1}) significantly reversed the scopolamine-induced impairment in freezing behaviour 24 hours post-training, with a 63% increase in freezing behaviour compared to the saline + scopolamine shock group ($p \leq 0.05$, Figure 2-11). Fear extinction occurred in both scopolamine + donepezil groups (1 and 2 mg kg^{-1}), scopolamine + donepezil (2 mg kg^{-1}) had a slower rate of extinction than that of scopolamine + donepezil (1 mg kg^{-1}) eliciting a small freezing response at 96 hours post-training. This experiment illustrates that the scopolamine-induced deficit can be reversed with pre-training administration of donepezil.

2.4 Discussion

The aim of this chapter was to optimise a CER paradigm in drug naïve rats, which would produce a robust freezing response when memory retention was tested. This was achieved by altering the training and testing protocol that caused rats to elicit a sub-maximal freezing response. Another aim was to alter freezing behaviour via administration of pharmacological agents, attenuation of freezing was induced with scopolamine and this was reversed following treatment of donepezil.

CER is commonly used to study fear and anxiety as well as an animal model of associative learning, many groups use CER to analyse molecular pathways

involved in learning and memory. The current experiments utilised CER as a robust learning and memory paradigm, consisting of a single training session which induced a strong conditioned emotion response, such as freezing. To optimise CER in rats, prior to testing pharmacological agents, a series of studies with different protocols were performed on drug naïve subjects. Many studies have tried to optimise CER in rodents; it has been shown that the timing of the US administration during training is critical, with immediate administration causing an attenuated freezing response compared to a delayed administration (Fanselow, 1986; Frankland et al., 2004b). Fanselow (1986) concluded that the deficit in freezing behaviour following an immediate shock was due to a weak association between the context and shock. Taking the literature into account, the primary experiment tested the effect of increasing the number of CS-US associations administered during training following a 30 second delay after exposure to the dark chamber of the conditioning context. This study confirmed that re-exposing a rat to a context where it had previously received a CS-US pairing induced a freezing response, as has been shown in previous groups (Kim and Fanselow, 1992), increasing the number of CS-US pairings caused a greater freezing response 24 hours post-training. Emotional responses include fighting, freezing and fleeing, prior to shock treatment rats elicited normal behaviours such as exploration and grooming, post-shock rats elicited defensive behaviours such as freezing and attempted fleeing (Bolles, 1970). Throughout some of the studies rats reared during training and testing sessions; this could be perceived as trying to escape from the foot shock or context. It should be noted that, throughout all experiments the total cumulative freezing behaviour was recorded, unlike some other studies in which, after set time intervals, it is determined whether the

animal is freezing and a percentage calculated. This approach was taken so as to obtain quantifiable times for the behaviour rather than subjective scores so that a more robust parametric analysis could be applied to all subsequent data analysis. This study illustrated that 3 CS-US pairings induced an optimal sub-maximal freezing response 24 hours post-training, making the protocol ideal for subsequent studies involving pharmacological agents.

CER allows extinction to be analysed; fear extinction can be used as an animal model of fear inhibition in humans, which is beneficial for research into the psychological disorders such as post-traumatic stress disorders and panic attacks. This set of studies clearly showed fear extinction of memory did occur when rats were re-exposed to the training context with no further CS-US pairings administered, illustrated by a decrease in freezing behaviour with each subsequent trial which is similar to other groups results (Tronson et al., 2008). This supports the theory that extinction is relearning, so the excitatory CS-US association formed during conditioning is not erased but rats learn a new inhibitory CS with no US association during extinction. There are three effects that support the view that extinction is relearning, these are renewal, spontaneous recovery and reinstatement. Renewal is the process where the learned freezing behaviour will occur if a CS is presented outside the context where extinction was performed; spontaneous recovery is where the CER recovers after an amount of time; reinstatement occurs when the US is administered after the extinction process causing the CER to be elicited (Bouton et al., 2006; Ji and Maren, 2007).

In humans, fearful memories can last a substantial amount of time; as previously stated this memory can reoccur spontaneously leading to a relapse of anxiety disorders. Data obtained in the second study to determine the strength of memory retention in CER showed that a robust freezing response is elicited up to 96 hours post-training with no subsequent exposure to the context. Other studies have found that rats can remember a fearful context after 16 months (Gale et al., 2004), further supporting the view that CER produces a strong long-term memory. As anxiety disorders are very detrimental, much research is performed in this area and recent work has shown that fear memories can be erased following fear extinction (Pizzorusso, 2009) which will be of great benefit. These studies confirm that CER is an appropriate behavioural paradigm to study long-term retention and extinction of memories.

Irrespective of the CS delivered during the test trial rats will elicit a strong freezing response, this study illustrated that both context and cue administration 24 hours post-training induced a freezing response of over 65% of the total test period. Previous studies have shown that the CER elicited from either a context or light and tone CS, are identical although the underlying signalling pathways might be different (Phillips and Ledoux, 1992). This could be due to the differences in classical conditioning, for instance in contextual conditioning there is a continuous CS that is not limited to a single sensory modality, rather than cued conditioning where there is an explicit CS (Fendt and Fanselow, 1999; Phillips and Ledoux, 1992). It has been shown that contextual fear crucially relies upon the hippocampus, whereas the amygdala is also involved in context and cue fear conditioning (Phillips and Ledoux, 1992). Due to this, further

studies throughout this thesis used CER to analyse further the underlying changes within the hippocampus.

There is a large amount of evidence supporting a role of cholinergic neurotransmission in learning and memory and cognitive dysfunction in CNS disorders, with increases in ACh levels observed during pre-clinical behavioural paradigms (Pepeu and Giovannini, 2009). It has been well documented that administration of the muscarinic acetylcholine receptor antagonist, scopolamine, will induce a memory deficit in a variety of behavioural paradigms (Li et al., 1997; Lindner et al., 2006). Pre-training administration of scopolamine caused a significant attenuation of freezing behaviour, which was due to a modulation of the central cholinergic neurotransmission as no effect was observed with methyl scopolamine. This is similar to many previous studies on fear conditioning where pre-training, but not post-training, administration of scopolamine impaired memory (Anagnostaras et al., 1999b; Gale et al., 2001), not all studies have found impairment of contextual fear with pre-training scopolamine (Young et al., 1995), although it should be noted that the differences in behavioural protocols and drug doses may account for this apparent disparity. This study confirmed that the CER protocol was susceptible to attenuation by a muscarinic receptor antagonist.

To enhance memory of CER donepezil and galantamine were utilised due to their cognitive enhancing effects in different behavioural paradigms (Yuede et al., 2007), and as they are used in the treatment of AD. However, these drugs had no effect on CER-induced memory in normal healthy rats, donepezil caused a slight increase in freezing behaviour at each test trial, suggesting a slight enhancement

of cognition, although this was not statistically significant. Little literature demonstrates the effects of these AChEI on normal healthy adult rats, but studies have analysed their effects on aged and pharmacologically impaired subjects (Barnes et al., 2000; Hernandez et al., 2006). In associative memory task, inhibitory and passive avoidance (IA and PA), both compounds have been tested in hypoxia-induced memory deficits and were found to have cognitive enhancing effects (Dimitrova and Getova-Spassova, 2006). Slight differences between the drugs were observed in the different tasks and it has been suggested that this may be due to different mechanisms of action, as galantamine is also an allosteric modulator of the nicotinic acetylcholine receptor. Supporting the role of the cholinergic neurotransmission in learning and memory, donepezil was found to reverse the attenuation of freezing behaviour following treatment with scopolamine. This reversal was only observed during the 24 hour retention trial, and is consistent with other studies that have analysed the ability of donepezil to reverse a scopolamine-induced deficit in fear conditioning (Lindner et al., 2006). Other chapters will utilise the scopolamine-induced deficit of CER to analyse the cognitive effects of drugs that act upon the 5-HT_6 receptor as studies have shown modulation of cholinergic neurotransmission following administration of 5-HT_6 receptor antagonists (Riemer et al., 2003).

2.5 Key Findings

This chapter has illustrated that changes in the CER protocol can alter the magnitude of the US-CS behaviour elicited in drug naïve rats. Once the optimal protocol was determined CER induced a strong robust memory that could be

retained up to 96 hours, and which would undergo extinction. Initial pharmacological studies showed that cholinergic neurotransmission plays a role in learning and memory in CER, as pre-treatment with scopolamine caused an attenuation in freezing which could be reversed by donepezil. Following these initial pharmacological studies the effects of 5-HT_6 receptor ligands can be analysed in CER.

3 The effect of a 5-HT$_6$ receptor antagonist, SB-271046, on conditioned emotion response

3.1 Introduction

Different stages of the learning and memory process are affected in CNS disorders. For example in schizophrenia there is a deficit in working memory, whereas AD causes an inability to form new memories. CER allows memory acquisition, consolidation and retention to be analysed in experimental animals in a single training trial by administering drugs at various stages throughout training and testing.

Cognitive dysfunction in CNS disorders, such as AD, is related to a decline in cholinergic neurotransmission (Perry et al., 1978); therefore, treatments to ameliorate this effect are constantly being researched. Administration of 5-HT$_6$ receptor directed antisense oligonucleotides (AO) and antagonists to rodents has a modulatory effect upon cholinergic neurotransmission; increasing ACh levels, demonstrated initially via an increase in the behavioural syndrome of stretching was later confirmed by *in vivo* microdialysis (Bourson et al., 1995; Riemer et al., 2003). In pre-clinical behavioural paradigms, 5-HT$_6$ receptor antagonists can reverse memory impairments induced via blockade of the muscarinic acetylcholine receptor (Foley et al., 2004; Mitchell and Neumaier, 2008; Woolley

et al., 2003). Although there is evidence that 5-HT_6 receptor antagonism can enhance cognition by increasing cholinergic neurotransmission, one group illustrated that the pro-cognitive effects of these antagonists on natural forgetting in the MWM may be induced via a non-cholinergic mechanism as the AChEI, donepezil (Aricept ™), had no effect in this task (Rogers and Hagan, 2001). Other groups have found that 5-HT_6 receptor antagonists can reverse glutamatergic-induced memory deficits (King et al., 2004; Pitsikas et al., 2008), and *in vivo* microdialysis studies confirm that these antagonists increase glutamate levels in the frontal cortex and dorsal hippocampus (Dawson et al., 2000, 2001), both areas associated with cognition. Taken together, these findings suggest that the cognitive enhancing effect of 5-HT_6 receptor antagonists may occur via an enhancement of cholinergic and glutamatergic neurotransmission.

The current chapter, therefore, investigates the acute effects of the 5-HT_6 receptor antagonist, SB-271046 (Bromidge et al., 1999), at different stages of the learning and memory process in a CER paradigm. The effect of the antagonist was examined initially when given alone, to determine any cognitive enhancing effects in normal subjects, and then the ability of SB-271046 to reverse a cholinergic and glutamatergic memory deficit was evaluated.

3.1.1 Cholinergic neurotransmission in cognition

Acetylcholine was the first neurotransmitter to be discovered, it exerts its effects upon two subtypes of receptors, nicotinic and muscarinic. Cholinergic neurons are found in the basal forebrain within the rat and project to the neocortex and hippocampus, supporting the role of cholinergic neurotransmission in learning

and memory. Cognitive decline in the elderly and in CNS disorders such as AD is associated with a decrease in cholinergic neurotransmission, with decreased cholinergic markers found within the cerebral cortex of Alzheimer's patients (Bartus et al., 1982; Perry et al., 1978). Due to the cholinergic hypothesis of cognitive decline in such CNS disorders, much research has focussed on drugs affecting cholinergic neurotransmission including their effects on attention, learning and memory using brain lesioning and pharmacological studies (Everitt and Robbins, 1997). Scopolamine (hyoscine) is a naturally occurring muscarinic receptor antagonist, like atropine, it is an alkaloid found within a group of plants including Atropa belladonna (Figure 3-1).

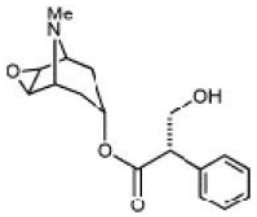


Figure 3-1- Structure of scopolamine (hyoscine)

Scopolamine binds to and antagonises the muscarinic acetylcholine receptor (of all subtypes), a member of the GPCR superfamily, causing a reduction in cholinergic neurotransmission; it has been used for the treatment of motion sickness and nausea. Pre-clinically scopolamine has been utilised by many groups to induce and model memory impairments in behavioural paradigms (Lindner et al., 2006; Mishima et al., 2000; Sunderland et al., 1986). As a further control to determine its specificity, administration of methyl scopolamine, which cannot cross the BBB, is frequently used to test the proposed site of the central

cholinergic effect rather than impairment of visual acuity, upon cognitive tasks. Previous groups have analysed the effects of scopolamine in Pavlovian conditioning (Anagnostaras et al., 2001; Anagnostaras et al., 1999b), illustrating that the pre-, but not post-training administration caused an impairment in the associative learning task. The results were slightly different in the passive avoidance paradigm where administration of scopolamine caused memory deficits when administered 6 hours post-training (Foley et al., 2004). As shown in Chapter 2 pre-training administration of scopolamine caused an attenuation of CER-induced freezing due to blockade of central cholinergic neurotransmission at muscarinic receptors, as methyl scopolamine had no effect on 24 hour retention. This is consistent with other studies analysing the effects of scopolamine on contextual fear conditioning (Lindner et al., 2003). Due to the accumulating evidence that decreased cholinergic neurotransmission impairs behavioural tasks, research focussed on those drugs that increase ACh levels, such as donepezil, an AChEI, approved for the treatment of cognitive decline in dementia and AD (Dooley and Lamb, 2000). There are concerns that the therapeutic effects of donepezil are small, for instance one study found that treatment of Alzheimer's patients with donepezil caused only a small improvement in cognitive tests compared to placebo treated controls and no effect upon behavioural and psychological symptoms (Courtney et al., 2004). Some groups believe that donepezil may only ameliorate the cognitive dysfunction in simple conditioning tasks. Lindner et al (2006) tested its effects in a battery of pre-clinical behavioural paradigms to measure attention, classical conditioning, working memory and spatial mapping. Results from this study found donepezil to have different effects upon the various cognitive tasks, with

the smallest effects upon higher function cognitive tasks (Lindner et al., 2006); therefore, new drugs are required to further aid cognitive dysfunction. As scopolamine is known to induce a robust impairment in CER (Chapter 2), the evidence that a 5-HT_6 receptor antagonist increased ACh levels (Riemer et al., 2003), and the literature that various antagonists can ameliorate cognitive impairments induced following muscarinic antagonism (Fone, 2008), the effects of SB-271046 on a scopolamine-induced impairment in CER were investigated in this chapter.

3.1.2 Glutamatergic neurotransmission in cognition

Glutamate is an excitatory neurotransmitter, it is a non-essential amino acid and is, therefore, readily synthesised within the body. There are two types of excitatory amino acid receptor, ionotropic (ion channel) and metabotropic (GPCR); the former is further subdivided into three, kainate, α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors. Activation of NMDA receptors causes an increase in intracellular Ca^{2+} levels, leading to activation of a number of molecular signalling pathways and activation of transcription factors; NMDA receptor activation is required for synaptic strengthening, LTP (see Chapter 2), a phenomena that has been linked to the underlying molecular pathways in learning and memory (Bear, 1997; Bliss and Collingridge, 1993). NMDA receptors are found within the hippocampus (Olverman et al., 1984) an area known to play a role in learning and memory of CER. In support of the role of glutamatergic neurotransmission in cognition, treatment with the NMDA antagonist aminophosphonovaleric acid (AP5) blocked hippocampal LTP (Morris et al., 1986), and NMDA receptor

antagonists impair memory in pre-clinical cognitive behavioural tasks. MK-801 ((+)-5-methyl-10, 11-dihydro-5Hdibenzo[a, d]cyclohepten-5, 10-imine maleate, dizocilpine, Figure 3-2) is a non-competitive NMDA receptor antagonist and has been suggested to produce several symptoms in animals similar to the psychosis and cognitive dysfunction seen in schizophrenia. For instance MK-801 produces cognitive deficits in a variety of pre-clinical behavioural tasks (Csernansky et al., 2005; King et al., 2004)

.

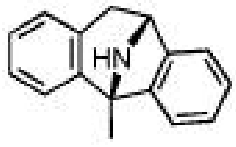


Figure 3-2-Structure of MK-801

Groups have analysed the stages of learning and memory that are affected by MK-801, it has been shown that in the novel object recognition task pre-training administration of MK-801 caused a deficit but this was not observed if the drug was administered immediately following the familiarisation trial or prior to the choice trial (Nilsson et al., 2007). This study suggests that NMDA receptors are required for acquisition/encoding of recognition memory and possibly consolidation rather than retrieval. One group found that post-training injections of MK-801 directly into the amygdala caused a dose-dependent decrease in memory retention when given alone and, in a separate study, MK-801 had exactly the same effect when administered into the dorsal hippocampus (Jafari-Sabet, 2006a, b). These studies support the theory of glutamatergic interactions in learning and memory in both the amygdala and hippocampus. In associative

learning tasks such as passive avoidance and contextual fear conditioning (CFC), pre-training administration of MK-801 caused significant memory deficits (Csernansky et al., 2005; Ishiyama et al., 2007; Jones et al., 1990). As glutamatergic neurotransmission plays a role in cognition and is increased in areas of the brain crucial for learning and memory following 5-HT_6 receptor antagonist treatment (Dawson et al., 2000, 2001), MK-801 was used to induce an impairment in CER and to test the ability of SB-271046 to reverse this effect.

3.1.3 5-HT_6 receptor antagonists on CER

As previously stated in Chapter 1, 5-HT_6 receptor antagonists have been shown to enhance learning and memory in many paradigms, such as MWM and NOR (Fone, 2008). One study utilised the NOR task to determine the stage(s) of learning and memory that were affected by administration of Ro 04-6790 and SB-271046. The study showed that 5-HT_6 receptor antagonism alleviated the memory deficit induced from a 4 hour inter-trial interval, if it was administered prior to, or immediately following, training (King et al., 2004). The experiments described in this current chapter employed a similar protocol to determine the stage(s) cognition affected by SB-271046 (Figure 3-3) on associative learning in CER using the same doses as validated in NOR.

Little literature is available about the effects of 5-HT_6 receptor antagonists on CER, one study analysed the effects of pre-training administration of either Ro 04-6790 (-30 minutes) or SB-271046 (-90 minutes) in conjunction with the muscarinic receptor antagonist, scopolamine on CFC, a very similar fear-motivated paradigm. Neither 5-HT_6 receptor antagonist ameliorated the

attenuation caused by scopolamine and it actually caused a reduction in freezing behaviour (Lindner et al., 2003). This is contradictory to studies that have utilised the passive avoidance paradigm, which, as stated previously, is another fear-motivated behavioural task, in which 5-HT_6 receptor antagonists ameliorate the attenuation induced by scopolamine (Foley et al., 2004).

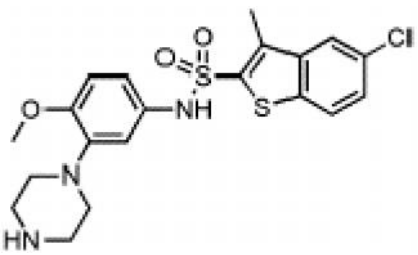


Figure 3-3-Structure of SB-271046

As stated in Chapter 2, cholinergic and glutamatergic neurotransmission play a role in CER, and administration of either scopolamine or MK-801 causes memory deficits in fear conditioning paradigms (Anagnostaras et al., 1999b; Csernansky et al., 2005). In other pre-clinical cognitive paradigms 5-HT_6 receptor antagonists have ameliorated the memory impairments induced from these compounds (Boast et al., 1999; Foley et al., 2004; King et al., 2004), and increased ACh and Glu levels within the brain (Dawson et al., 2000, 2001; Riemer et al., 2003). Therefore, 5-HT_6 receptor antagonists may ameliorate cholinergic and glutamatergic induced memory impairments in associative learning.

3.1.4 Aims

The aims of the current chapter were to determine the effect of acute administration of the 5-HT_6 receptor antagonist, SB-271046, on CER, this was achieved by:

- Determining the stage of learning and memory in CER that is affected by SB-271046 by varying the drug administration time during CER training and testing.
- Analysing the effect of SB-271046 on drug-induced cholinergic and glutamatergic memory deficits in CER.

3.2 Methods

3.2.1 Animals

Adult male Lister Hooded rats (University of Nottingham BMSU, derived from Charles River stock, or Charles River UK) were housed in groups of 3-6. Rats were kept under the same standard conditions described in Chapter 2, section 2.2.1.

3.2.2 Drugs

SB-271046 (5-chloro-N-(4-methoxy-3-piperazin-1-yl-phenyl)-3-methyl-2-benzothiophenesulfonamide, Figure 3-3) was provided by GlaxoSmithKline. Scopolamine hydrobromide and (+)-MK-801 maleate (dizocilpine) were purchased from Sigma Aldrich (Dorset, UK). SB-271046 was dispersed in 0.154M saline containing 0.5% (w/v) methylcellulose. Scopolamine and (+)-

MK-801 were dissolved in sterile physiological saline. SB-271046 and corresponding vehicle control were delivered in 3 ml kg^{-1} volume (determined from previous experiments in our laboratory, (King et al., 2004)), scopolamine, MK-801 and saline controls were delivered in 1 ml kg^{-1} i.p.

3.2.3 Conditioned Emotion Response

The apparatus and behaviour training and testing protocols performed throughout this chapter follow those described in Chapter 2 section 2.2.3 and 2.2.4.

3.2.4 Experiment I: Effect of SB-271046 on acquisition, consolidation and retention of memory in CER

Three separate experiments were performed to determine the effects of varying the administration point of the 5-HT_6 receptor antagonist, SB-271046, on memory acquisition, consolidation and retention in CER. To test memory acquisition rats, weighing 230-270g, were randomly assigned into one of four groups (n=7-8); vehicle + no shock; vehicle + shocked; SB-271046 + no shock and SB-271046 + shocked. SB-271046 was administered to rats that received no shock to establish any confounding effects upon CER. To test memory consolidation and retention, two separate groups of rats (weighing 240-300g, n=8-9, and 280-310g, n=9-11, accordingly) were assigned into one of three treatments; vehicle + no shock; vehicle + shocked and SB-271046 + shocked. SB-271046 was not administered to non-shocked animals as it did not produce an effect in this group when administered prior to conditioning; this avoided any unnecessary use of animals. On the training day, for memory acquisition and

consolidation experiments each rat received SB-271046 (10 mg kg $^{-1}$) or corresponding vehicle at one of two time points: 30 minutes prior to training or immediately following training, to test memory retention SB-271046 (10 mg kg^{-1}) was administered 30 minutes prior to 24 hour retention trial. Freezing behaviour was measured 24-96 hours post-training.

3.2.5 Experiment II: Effect of SB-271046 on a scopolamine-induced cognitive deficit

Rats, weighing 240-300g, were randomly assigned into one of five treatment combination groups (n=6-8); saline + vehicle + no shock; saline + vehicle + shocked, scopolamine + vehicle + shocked, scopolamine + SB-271046 (10 mg kg^{-1}) + shocked, and scopolamine + SB-271046 (15 mg kg^{-1}) + shocked. One group of rats received scopolamine + vehicle, with foot shock, in order to clarify the effects of scopolamine within this individual experiment. Saline (1 ml kg^{-1}, i.p.) or scopolamine (0.3 mg kg^{-1}, i.p., dose determined from previous chapter and Anagnostaras et al 1999b) were administered 20 minutes prior to training. Immediately following training, rats were dosed with vehicle (0.5% methylcellulose, 3 ml kg^{-1}, i.p.) or SB-271046 (10 mg kg^{-1} or 15 mg kg^{-1}, i.p.) accordingly.

3.2.6 Experiment III: Effect of SB-271046 on a MK-801-induced cognitive deficit

Rats were randomly assigned into one of four treatment combination groups (weighing 240-380g, n=8-10); saline + vehicle + no shock; saline + vehicle +

shocked, MK-801 + vehicle + shocked, and MK-801 + SB-271046 + shocked. To determine any confounding effects that MK-801 may have had on CER, a separate group of rats received MK-801 and vehicle with foot shock. Saline (1 ml kg^{-1}, i.p.) or MK-801 (0.1 mg kg^{-1}, i.p.) were administered 20 minutes prior to training. Immediately following training, rats were dosed with vehicle (0.5% methylcellulose, 3 ml kg^{-1}, i.p.) or SB-271046 (10 mg kg^{-1}, i.p.) accordingly. The dose of MK-801 was chosen based on a previous CER validation experiment (data not shown) and the existing literature (Csernansky et al., 2005; Nilsson et al., 2007).

3.2.7 Measured variables and Statistical Analyses

During CER training the latency to cross into the dark chamber was automatically recorded by the ShutAvoid program. Total cumulative time spent freezing was recorded manually throughout each retention and extinction trial by an observer blind to treatment.

Latency times were analysed using an ANOVA; between-conditions analysis was made on the drug treatment administered.

When retention and extinction trials were recorded (experiment I, effects of SB-271046 on acquisition and consolidation) repeated measures ANOVA followed by Tukey's post-hoc was utilised to determine the effect of shock and drug treatment on the freezing response elicited. A two-way ANOVA was used to determine the effect of drug and shock on CER-induced freezing in experiment I, effect of SB-271046 on acquisition, during the 24 hour retention trial alone.

To determine the difference between shock and no shock condition rats in experiments IB, IC, II and III a Student's *t*-test was performed between the no shock and shocked vehicle-treated groups. In addition, between-conditions analysis of drug treatment was determined using either a one-way ANOVA with Tukey's post-hoc, on all shock treated groups (if more than two), or Student's *t*-test (with two groups only, experiment IB and IC).

3.3 Results

3.3.1 Experiment I: Effect of SB-271046 on acquisition, consolidation and retention of memory in CER

SB-271046 administered 30 minutes prior to CER training had no effect on the time taken to cross into the dark chamber on the training day (Table 3-1A, $p>0.05$). Treatment with SB-271046, 30 minutes prior to the training trial, significantly attenuated CER-induced freezing behaviour in the 24 hour retention trial (54% reduction). Initially, freezing times from all extinction tests were analysed (Figure 3-4), repeated measures ANOVA did not indicate a time x shock interaction, although there was a significant effect of shock $F_{(1,26)}=20.074$, p=0.001. Between-conditions ANOVA, combining shock and drug treatment as the factors, revealed significance $F_{(15,119)}=13.899$, p=0.001. Freezing behaviour elicited by shocked vehicle-treated rats at the 24 hour retention test was significantly longer than all other groups at that particular test time (219.8 ± 21.7 s, $p\leq0.001$, Figure 3-4), SB-271046 + shock-treated rats also froze significantly longer than the two no-shock control groups ($p\leq0.01$, Figure 3-4) but the

(A)

Treatment	Latency (secs)
Vehicle NS	10.1 ± 1.6
SB-271046 NS	13.6 ± 3.0
Vehicle	13.4 ± 2.9
SB-271046	18.9 ± 6.0

(B)

Treatment	Latency (secs)
Vehicle NS	9.7 ± 1.6
Vehicle	11.1 ± 1.8
SB-271046	10.9 ± 2.0

(C)

Treatment	Latency (secs)
Vehicle NS	14.3 ± 2.4
Vehicle	8.4 ± 1.5
SB-271046	9.6 ± 1.6

Table 3-1- Latency times for experiments on effects of SB-271046 administered at various times throughout CER training and testing. No effects were observed between groups on latency to cross into the dark period on training day in experiments where SB-271046 was administered alone. Average time (seconds, mean ± s.e.m) for rats to cross from the light to dark chamber during training session when SB-271046 was administered (A) 30 minutes prior to training (n=7-8), (B) immediately following training (n=8-9), and (C) 30 minutes prior to testing (n=9-11). No statistical differences were observed between groups within each experiment. NS = No shock.

Figure 3-4- SB-271046 administered prior to CER training reduced CER-induced freezing behaviour. Average time spent freezing (seconds, mean ± s.e.m) during each test trial, four groups of rats received a single injection of vehicle (0.5% methyl cellulose, 3 ml kg^{-1}, i.p.) or SB-271046 (10 mg kg^{-1}, i.p.) 30 minutes prior to CER training with or without foot shock. Two-way repeated measures ANOVA revealed significant effect of shock $F_{(1,26)}$=20.074, p=0.001, Tukey's post-hoc following ANOVA (combining shock and drug treatment) revealed a significant effect $F_{(15,119)}$=13.899, p=0.001, *** *p≤0.001* and ** *p≤0.01* versus vehicle NS during same testing trial, ### *p≤0.001* and ## *p≤0.01* versus SB-271046 NS during same testing trial, ††† *p≤0.001* versus vehicle shock during same testing trial. There was statistical significance observed between the groups at extinction trials and the 24 hour shocked vehicle and SB-271046 groups (data not shown).

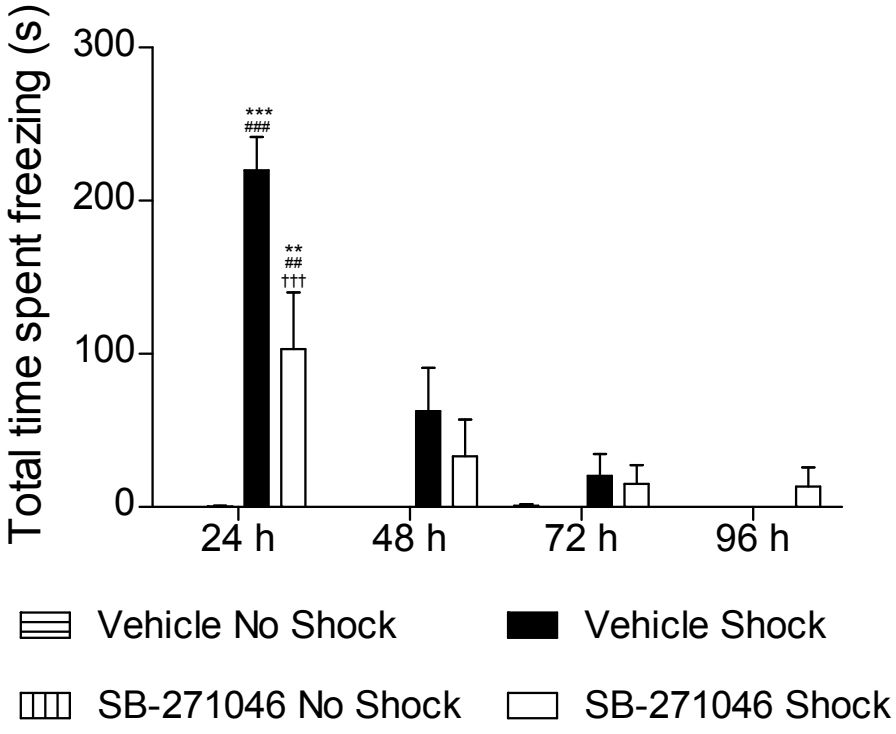
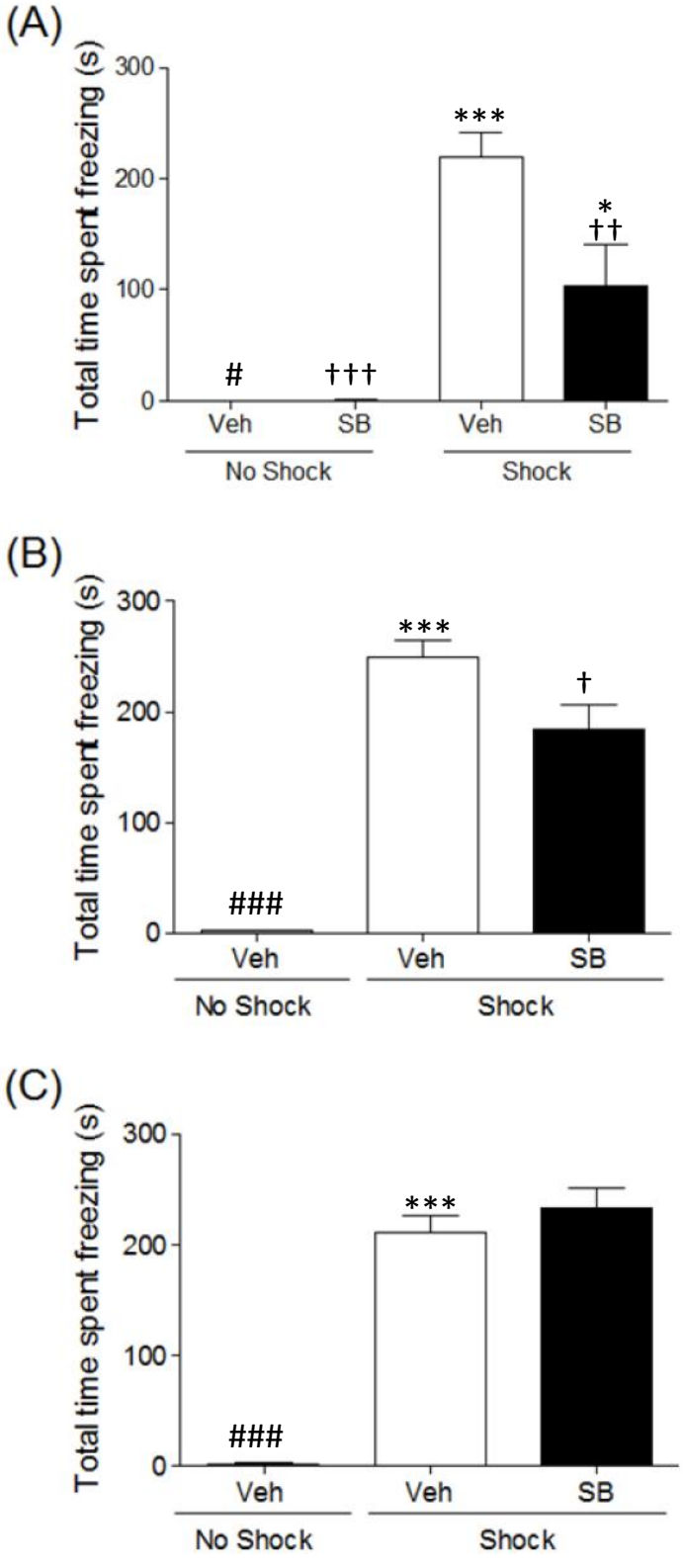

Total time spent freezing (s)
300
200
100
0
***
###
**
##
†††
24 h
48 h
72 h
96 h
Vehicle No Shock
Vehicle Shock
SB-271046 No Shock
SB-271046 Shock

Figure 3-5- Administering the 5-HT_6 receptor antagonist, SB-271046, at different stages of learning and memory in CER had differing effects on the freezing behaviour induced 24 hours post-training (seconds, mean ± s.e.m). Three separate groups of rats received SB-271046 (10 mg kg^{-1}, i.p.) either (A) 30 minutes prior to training (n=7-8), (B) immediately after training (n=8-9) and (C) 30 minutes prior to 24 hour retention test (n=9-11). *** *p≤0.001*; * *p≤0.05* versus own no shock control group, ††† *p≤0.001*; †† *p≤0.01*; † *p≤0.05* versus vehicle shock group, ### *p≤0.001*; # *p≤0.05* versus SB-271046 shock group. SB = SB-271046, veh = vehicle.


(A)
Total time spent freezing (s)
300
200
100
0
***
*
††
#
†††
Veh
SB
Veh
SB
No Shock
Shock
(B)
Total time spent freezing (s)
300
200
100
0
***
†
###
Veh
Veh
SB
No Shock
Shock
(C)
Total time spent freezing (s)
300
200
100
0
***
###
Veh
Veh
SB
No Shock
Shock

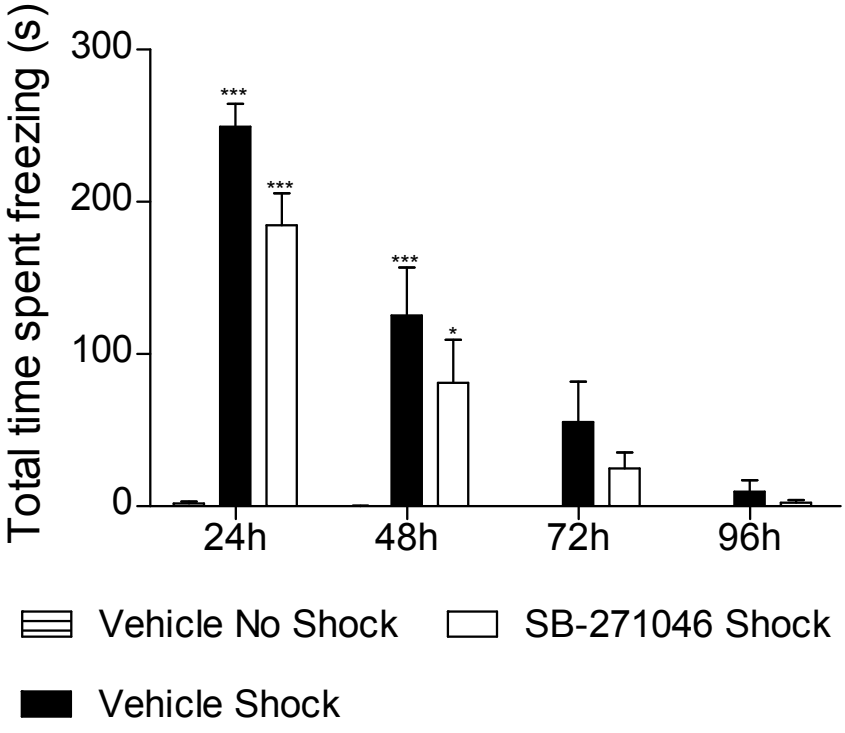
behaviour was attenuated in comparison to the vehicle + shock treated group. Both no-shock control groups froze for less than 1% of the total time tested at each post-conditioning day examined, showing that rats that did not receive a foot shock, did not find CER apparatus aversive. Extinction of memory clearly occurred in the vehicle and SB-271046 + shock-treated rats. In both shock-treated groups, freezing behaviour reduced with every extinction trial performed compared to that of the previous test period (with significance in vehicle shock treated rats between 24 hour retention trial and all three subsequent tests, $p \leq 0.001$, and in SB-271046 shock treated rats between 24 hour retention trial and 72 ($p \leq 0.05$) and 96 ($p \leq 0.01$) hour extinction trials, Figure 3-4). At the 96 hour extinction test, vehicle and SB-271046 shock-treated groups exhibited freezing behaviour of similar magnitude to the no shock control groups, less than 5% of total test period (Figure 3-4).

Subsequent studies focussed on the 24 hour retention trial; therefore, these data points alone were analysed in this experiment (Figure 3-5A). Overall, the statistics were the same as analysing all time points (24-96 hours), only slight differences in p values were observed in the Tukey's post-hoc test. Between-conditions ANOVA showed a significant shock x drug treatment interaction ($F_{(1, 30)} = 6.45$, $p = 0.017$), vehicle + shock-treated rats froze significantly longer than all other groups ($p \leq 0.001$ versus both no-shock control groups, $p \leq 0.01$ versus SB-271046 + shock group), and the SB-271046 + shock-treated rats froze significantly longer than the no-shock control groups ($p \leq 0.05$, Figure 3-5A).

As this was the initial study with the 5-HT_6 antagonist in CER, the effects of SB-271046 without shock exposure was tested to determine if the compound had any adverse effects upon CER-induced freezing procedure. No statistical difference was noted between the vehicle and SB-271046 + no-shock-treated groups (*$p>0.05$*) and subjective observation failed to show any signs of abnormal behaviour, illustrating that SB-271046 was having no confounding effects on CER. Due to this reason, subsequent studies did not include a SB-271046, no-shock group to comply with the 3Rs, and focussed on the effects of these drugs on shock-treated rats. This caused uneven group numbers in subsequent experiments; therefore the effect of shock was determined with between-conditions analysis of no-shock and shock vehicle-treated rats, and effects of drug upon CER were determined with between-groups analysis of all shock-treated groups.

The second study analysed the effects of administering SB-271046 immediately following CER training; freezing behaviour was recorded daily, 24-96 hours post-training. As expected, since no drug treatment was given prior to training, no differences were observed between groups in time to cross into the dark period during CER conditioning (Table 3-1B). As with the first study in this drug-induced alteration type of experiment initially all test trials were analysed (24-96 hours), showing a significant main effect of treatment (combining shock and drug treatment) with a repeated measures ANOVA $F_{(2,23)}=26.910$, $p=0.001$ (Figure 3-6). Further analysis with a Tukey's post-hoc following ANOVA revealed that shock-treated rats, independent of vehicle or SB-271046 administration, froze significantly longer than the no-shock-treated group at both 24 (*$p\leq0.001$*) and 48

Figure 3-6- No effect on CER-induced freezing behaviour was observed when the 5-HT_6 receptor antagonist SB-271046 was administered immediately following training (memory consolidation) over 24-96 hours post-conditioning. Average time spent freezing (seconds, mean ± s.e.m) during each test trial, four groups of rats received a single injection of vehicle (0.5% methyl cellulose, 3 ml kg^{-1}, i.p.) or SB-271046 (10 mg kg^{-1}, i.p.) immediately following CER training. Repeated measures ANOVA revealed significant effect of treatment (combining shock and drug treatment) $F_{(2,23)}$=26.910, p=0.001 Tukey's post-hoc revealed further significance *** $p \leq 0.001$ and * $p \leq 0.05$ versus vehicle no-shock-treated rats during same testing trial. There was statistical significance observed between the groups at extinction trials and the 24 hour shocked vehicle and SB-271046 groups (data not shown).


Total time spent freezing (s)
300
200
100
0
***
***
***
*
24h
48h
72h
96h
Vehicle No Shock
SB-271046 Shock
Vehicle Shock

($p \leq 0.001$ and $p \leq 0.05$ respectively) hour testing trials. Drug treatment had no effect upon freezing time during any test trials, with no statistical significance observed between the two shocked-treated groups at any time point tested. This experiment illustrates clear extinction of the memory with each test trial, both shock treated groups exhibited a decreased freezing behaviour with each subsequent test performed, suggesting that the rats were relearning the context was no longer an aversive environment. By the final testing trial, 96 hours post-conditioning, both shocked-treated groups froze for less than 5% of the total testing period; this is of similar magnitude to the no-shock-treated control group.

When 24 hour retention data alone were analysed, a significant effect of drug treatment was observed, as there were only two drug groups a Student's *t*-test was performed to determine independent effects of shock and drug treatment on CER-induced freezing behaviour. Analogous to findings with repeated measures ANOVA the shock had a major effect on freezing behaviour, $p \leq 0.001$. Student's *t*-test found treatment with SB-271046 caused a small but significant reduction (26%) in freezing behaviour (184.5 ± 21.5 s) compared to the vehicle + shock group (249.3 ± 15.1 s, Figure 3-5B, $p \leq 0.05$).

Finally, the effect of SB-271046 administered 30 minutes prior to the retention test (24 hour post-conditioning) was analysed. As expected, since no drug treatment was given during prior to conditioning, the latency for each different treatment group of rats was unaltered (Table 3-1C). No effect on CER-induced freezing behaviour was observed during 24 hour retention trial when SB-271046 was administered 30 minutes prior to testing period. There was no difference in

time spent freezing between the two shock-treated groups (Student's *t*-test) during the 24 hour retention test, both the vehicle and SB-271046 groups that received shocks froze for over 70% of the total test period (SB-271046 + shock-treated rats froze for 233.7 ± 17.3 s and vehicle + shock-treated group froze for 210.3 ± 15.7 s, $p>0.05$, Figure 3-5C). As with previous studies the shock had a significant effect upon freezing behaviour, with both shock treated groups freezing significantly longer than the no shock control group ($p\leq0.001$), which froze for less than 1% of the test period (Figure 3-5C). SB-271046 did not have any effect on CER-induced freezing behaviour when administered 30 minutes prior to the retention test, suggesting that 5-HT_6 receptor antagonists have no effect on the memory retention process in CER in normal animals.

From these three studies it is evident that the 5-HT_6 receptor antagonist, SB-271406, appears to exert its effects upon memory acquisition and consolidation in CER (although see further discussion of this apparent effect later in the discussion section). Significant attenuation of freezing behaviour was elicited when SB-271046 was administered prior to training; this was reduced with administration immediately post-training. Due to this, and the evidence that SB-271046 has little effect on memory retention in this paradigm, for all subsequent studies SB-271046 was administered immediately post-training.

3.3.2 Experiment II: Effect of SB-271046 on a scopolamine-induced cognitive deficit

This study examined the effect of post-training administration of SB-271046 on a scopolamine-induced memory deficit during the 24 hour retention test. Scopolamine was injected prior to training (-20 minutes) to prevent learning while SB-271046 or vehicle was given immediately following training. The injection prior to training did not produce any confounding effects upon the freezing response elicited with the saline + vehicle, shocked group freezing for a comparable amount of time to that observed in previous studies. The injection of scopolamine or saline prior to training had no significant effect (ANOVA) upon the latency to cross into the dark context (Table 3-2A). Similarly to previous studies, the saline + vehicle, no-shock control group froze for less than 1% of total test period, suggesting that there is no aversion to the chamber unless shock conditioning had been received. Thus, as expected, the saline + vehicle shocked group froze significantly longer than the saline + vehicle no shock control group ($p \leq 0.001$, Student's *t*-test Figure 3-7). To determine the effect of drug treatment on freezing behaviour a one-way ANOVA was performed on all the groups which received shock; this revealed a significant main effect of treatment between all shock-treated rats ($F_{(3,30)}=12.422$, $p=0.001$). To ensure scopolamine was causing a cognitive deficit in CER, one group of rats received scopolamine + vehicle, as with previous experiments (Chapter 2 Section 2.2.4.4), pre-training administration (-20 minutes) of scopolamine attenuated CER-induced freezing behaviour compared to the shocked vehicle group ($p \leq 0.001$, Figure 3-7) during the 24 hours post-conditioning test. Two doses of SB-271046 were administered in conjunction with scopolamine to attempt to accurately evaluate if the 5-HT_6

(A)

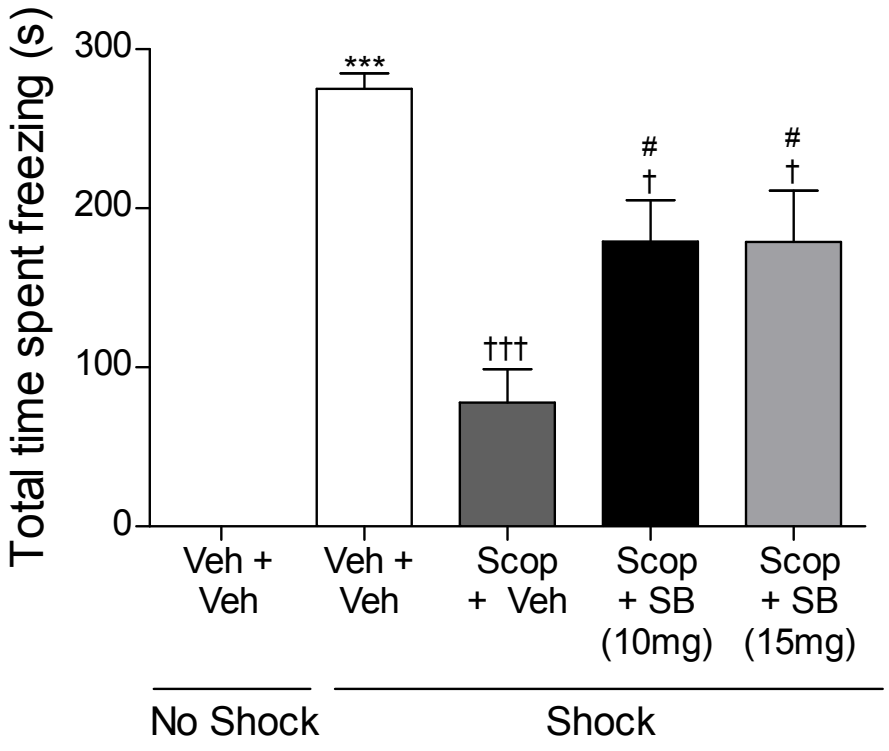
Treatment	Latency (secs)
Saline + Vehicle NS	8.8 ± 3.0
Saline + Vehicle	5.8 ± 0.9
Scopolamine + Vehicle	5.0 ± 1.7
Scopolamine + SB-271046 (10 mg kg^{-1})	8.4 ± 2.8
Scopolamine + SB-271046 (15 mg kg^{-1})	10.5 ± 2.9

(B)

Treatment	Latency (secs)
Saline + Vehicle NS	11.7 ± 0.7
Saline + Vehicle	17.6 ± 1.8
MK-801 + Vehicle	11.2 ± 2.3 *
MK-801 + SB-271046 (10 mg kg^{-1})	11.6 ± 1.2 *

Table 3-2- Average latency time (seconds, mean ± s.e.m) for rats to cross into the dark chamber during training session when testing the ability of SB-271046 to reverse either (A) scopolamine-induced or (B) MK-801-induced memory deficit in CER. No differences between groups were observed when rats were trained under the influence of scopolamine. One-way ANOVA revealed a slight difference between saline + vehicle shock and both groups receiving MK-801 in experiment testing effects of SB-271046 on MK-801-induced memory deficits $F_{(3,35)}= 3.796$, p=0.02, * $p \leq 0.05$ versus vehicle + vehicle (Tukey's post-hoc). NS = no shock.

Figure 3-7- Post-training administration of SB-271046 partially reversed a scopolamine-induced deficit of freezing behaviour in a CER paradigm during 24 hour retention trial (seconds, mean ± s.e.m). Rats were assigned to five groups (n=6-9), saline (1 ml kg^{-1}) or scopolamine (0.3 mg kg^{-1}) was administered 20 minutes prior to training, with vehicle (0.5% methyl cellulose, (3 ml kg^{-1}) or SB-271046 (10 or 15 mg kg^{-1}) given immediately following training. Student's *t*-test was performed to determine the effect of shock on freezing behaviour between vehicle no shock and shock treated groups *** $p \leq 0.001$ versus vehicle no shock control group. To determine effect of drug treatment on CER-induced freezing response between-conditions ANOVA was used, ($F_{(3,30)}$=12.422, p=0.001) followed by Tukey's post-hoc ††† $p \leq 0.001$; † $p \leq 0.05$ versus vehicle shock group, # $p \leq 0.05$ versus scopolamine shock group. SB = SB-271046, scop = scopolamine, veh = vehicle.

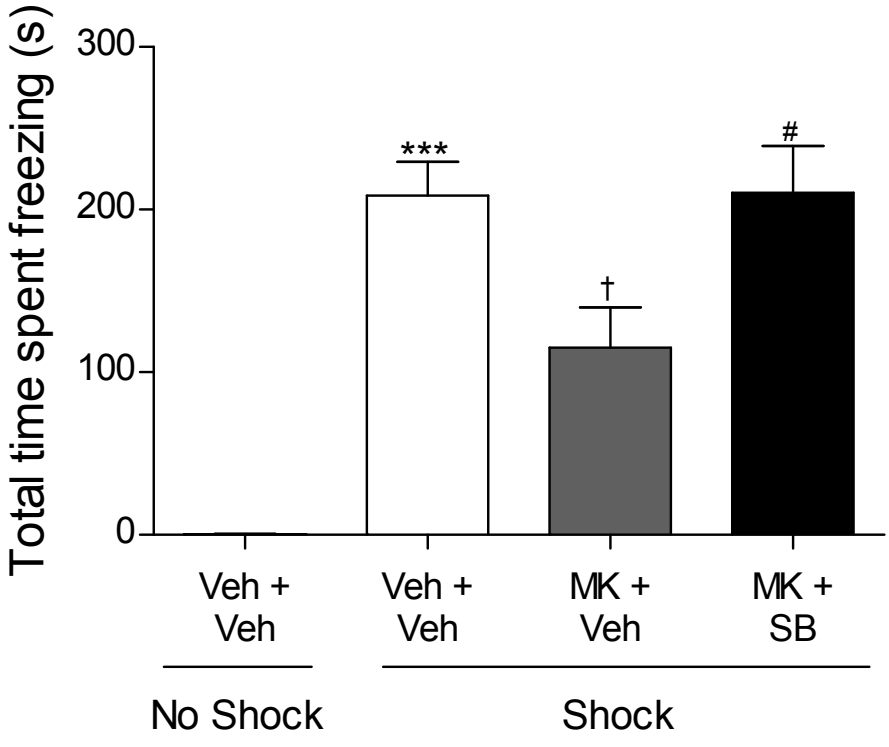

Total time spent freezing (s)
300
200
100
0
***
#
†
#
†
†††
Veh + Veh
Veh + Veh
Scop + Veh
Scop + SB (10mg)
Scop + SB (15mg)
No Shock
Shock

receptor antagonist could reverse the cognitive deficit induced. SB-271046, at both 10 and 15 mg kg^{-1}, partially reversed the scopolamine-induced cognitive deficits ($p \leq 0.05$), although both these groups froze significantly less than the saline + vehicle, shocked group ($p \leq 0.05$). The saline + vehicle shock-treated rats elicited a slightly higher freezing response within the current experiment, but each experiment had individual vehicle control groups and therefore, responses elicited are controlled for within a particular experiment. There were no differences between the two doses of SB-271046 tested ($p > 0.05$).

3.3.3 Experiment III: Effect of SB-271046 on a MK-801-induced cognitive deficit

The effects of SB-271046 on a glutamatergic-induced cognitive deficit were analysed in this experiment using a similar dosing protocol to that already explained for scopolamine. The saline + vehicle shocked-treated rats took significantly longer to cross into the dark chamber than both groups that received MK-801 (Table 3-2B, $p \leq 0.05$) and just under significance compared to vehicle no shock treated rats ($p = 0.55$) during CER training. This was due to 4 subjects (in the saline + vehicle, shocked group) spending over 20 seconds in the light chamber prior to crossing into the dark chamber; therefore increasing the average latency time for the group. MK-801 was not having an adverse effect upon the subjects as both groups that received the NMDA receptor antagonist took a similar amount of time to the second group which received saline prior to training (Table 3-2B). As with experiment II the shock treatment was required to cause freezing behaviour such that the saline + vehicle shock group froze for a

Figure 3-8- Post-training administration of SB-271046 reversed an MK-801-induced deficit on CER-induced freezing behaviour during the 24 hour retention trial of CER (seconds, mean ± s.e.m). Rats were assigned into four treatment combination groups (n=8-10), saline (1 ml kg^{-1}) or MK-801 (0.1 mg kg^{-1}) was administered 20 minutes prior to training, with vehicle (0.5% methyl cellulose, (3 ml kg^{-1}) or SB-271046 (10 mg kg^{-1}) immediately following training. Effect of shock on CER-induced freezing was analysed with a Student's *t*-test between vehicle shock and no shock treated rats *** $p \leq 0.001$ versus, vehicle no-shock control group. Between-conditions ANOVA followed with Tukey's post-hoc was utilised to determine effect of drug on shock treated CER-induced freezing response, $F_{(2,26)}=4.266$, p=0.026, † $p \leq 0.05$ versus vehicle shock group, # $p \leq 0.05$ versus MK-801 shock group. MK = MK-801, SB = SB-271046, veh = vehicle.


Total time spent freezing (s)
300
200
100
0
***
†
#
Veh +
Veh
Veh +
Veh
MK +
Veh
MK +
SB
No Shock
Shock

significantly longer time (208.5 ± 20.7s) than the saline + vehicle no-shock group (0.3 ± 0.3 s, $p \leq 0.001$, Student's *t*-test, Figure 3-8). An initial CER validation experiment (data not shown) determined the dose of MK-801 used in this, and subsequent experiments, and it determined that MK-801 had no confounding effect upon freezing behaviour elicited. Between-conditions analysis revealed an effect of drug treatment on freezing behaviour 24 hours post-training (ANOVA $F_{(2,26)}=4.266$, p=0.026), MK-801 administered 20 minutes prior to training attenuated freezing behaviour at the 24 hour retention trial compared to the saline + vehicle shock group ($p \leq 0.05$), causing a 45% reduction in freezing behaviour observed. Only one dose of SB-271046 was administered in conjunction with MK-801 for this experiment because no differences between two doses of SB-271046 administered were observed in the cholinergic deficit study. SB-271046 fully reversed the cognitive deficit observed following treatment with MK-801 ($p \leq 0.05$, Figure 3-8). No statistical difference was observed between freezing responses elicited by the saline + vehicle shock and MK-801 + SB-271046 shock treated groups, with both exhibiting a freezing behaviour greater than 69% of the total testing time. This experiment illustrates that SB-271046 can reverse a cognitive deficit in CER that is induced from pre-training administration of MK-801.

3.4 Discussion

The aim of the current chapter was to determine the stage(s) of the learning and memory process in CER that the 5-HT_6 receptor antagonist, SB-271046, altered.

Following on from this the aim was to elucidate if SB-271046 could reverse either cholinergic or glutamatergic memory impairments in this paradigm.

An array of pre-clinical behavioural studies have been performed using a variety of 5-HT_6 receptor antagonists that have illustrated their cognitive enhancing effects (Mitchell and Neumaier, 2005). It is apparent from the literature that the task used, administration time of the antagonist, and the age of the rats is crucial to the responses evoked. The present study has highlighted the stages of the learning and memory process that the selective 5-HT_6 receptor antagonist, SB-271046, modulates in CER by varying the administration time across the protocol. SB-271046 has an effect upon memory acquisition and consolidation in this associative task, as when it is administered 30 minutes prior to training there was a significant attenuation of freezing behaviour, this attenuation was reduced when administered immediately following training. There does not seem to be any effect of SB-271046 upon the retention process as no difference in freezing behaviour was observed when it was administered at the same dose 30 minutes prior to testing 24 hour post-conditioning. These findings were unexpected as many other pre-clinical cognitive paradigms have found SB-271046 to have a pro-cognitive effect. A similar experiment determined the stage of learning and memory in NOR that was affected with treatment of Ro-04-6790 and SB-271046, interestingly this found that administration prior to or immediately after the familiarisation trial, but not prior to the second trial, reversed a time-induced memory deficit (King et al., 2004). The current results, and those from literature, suggest that 5-HT_6 receptor antagonists affect memory consolidation but have differing cognitive effects in various pre-clinical behavioural tasks (Fone, 2008).

As previously stated, little literature to date is available demonstrating the effects of the 5-HT_6 receptor antagonist, SB-271046 on CER in healthy, drug-naïve subjects, but effects in passive avoidance, another fear-motivated behavioural paradigm, have been shown. It was found that acute administration of 5-HT_6 receptor antagonists (4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine, Ro-04-6790, SB-271046 have little effect when given alone, but have been shown to reverse a scopolamine-induced deficit in both young and old rats (Bos et al., 2001; Foley et al., 2004; Riemer et al., 2003), although not all groups have been able to replicate these findings (Lindner et al., 2003). In the present study the magnitude of the attenuation observed was much greater when SB-271046 was administered prior to, rather than after, training, which is analogous to the findings by Lindner and colleagues (2003) who showed an attenuation of freezing when rats were trained following administration of 5-HT_6 receptor antagonists. The inhibitory response, observed in the current experiment, could reflect impairment of memory acquisition; however this does not fit with the pro-cognitive effects demonstrated by this compound at this dose in a variety of other cognitive tasks. Therefore, an alternative explanation is that SB-271046 is actually impairing the CS-US association perhaps due to an anti-nociceptive or anxiolytic effect of the 5-HT_6 receptor antagonist (Finn et al., 2007; Wesolowska et al., 2007). Although there are contradictory findings of the effects of 5-HT_6 receptors on nociception and anxiety, see Chapter 1, section 1.3.5.11. CER employs mild foot shocks to form an association between the context and the cue (light, tone and shock), therefore administering SB-271046 prior to training could alter the perception of the foot shocks leading to a weaker association between the context and shock. This supports the data obtained in

this study as the attenuation in freezing behaviour was reduced (i.e. a stronger association between context and cue was formed) when SB-271046 was administered post-training and, hence, after the presentation of the foot shocks. As SB-271046 was having a negative effect upon cognition if administered prior to training it was determined for future studies it would be delivered immediately following training, to eliminate the possibility of an effect upon the formation of CS-US associations.

The current studies also importantly showed that SB-271046 can ameliorate deficits in learning and memory induced by the muscarinic acetylcholine antagonist, scopolamine, and the NMDA antagonist, MK-801. This study had similar findings to other reports demonstrating that SB-271046 can ameliorate the scopolamine-induced memory impairments in the passive avoidance paradigm (Foley et al., 2004). Lindner and colleagues (2003) found neither Ro 04-6790 or SB-271046 could reverse a scopolamine-induced memory deficit in CER, the differences between that study and the current one is timing of drug administration, Lindner et al (2003) administered 5-HT_6 receptor antagonists and scopolamine together prior to training (either -30 or -90 minutes), whereas scopolamine was administered prior to, and SB-271046 immediately following, training in the current study. Another observation from the study by Lindner and colleagues (2003) was the vehicle + no-shock control groups froze ~30% of test time which questions whether there were other affects (such as anxiety) masking those of CFC-induced freezing behaviour, as this is a lot longer than the equivalent behaviour in the vehicle no shock controls in the current experiments. Data obtained from the present study indicate an effect of SB-271046 in CER

which is sensitive to the activation of the cholinergic neurotransmission system. These findings support the theory that 5-HT_6 receptor antagonism has a modulatory role in cholinergic neurotransmission, initially hypothesised following evidence that administration of antagonists induced stretching behaviour in rats which was blocked with pre-treatment of scopolamine and atropine (Bentley et al., 1999; Lindner et al., 2003), and can reverse cholinergic deficits in various behavioural paradigms (Hirst et al., 2006; Lieben et al., 2005; Mitchell et al., 2006).

Activation of the NMDA receptor is known to be involved in learning, memory and LTP, consistent with this is the finding that blockade of this receptor by administration of the non-competitive NMDA antagonist, MK-801, induces a memory deficit in a variety of behavioural tasks, including fear conditioning (Boast et al., 1999; Csernansky et al., 2005; King et al., 2004). Supporting these previous findings the current study showed that pre-training treatment of MK-801 attenuated freezing behaviour following conditioning, suggesting glutamatergic neurotransmission plays a vital role in learning and memory of CER. Following MK-801 with SB-271046 treatment fully restored the freezing behaviour elicited in the 24 hour retention trial. Data obtained from this study suggest that SB-271046 may enhance memory acquisition and consolidation in CER by modulating central glutamatergic neurotransmission involved. Previous groups found that pre-training administration of MK-801 caused memory deficits in NOR when given alone, it also prevented the restoration of recognition memory following a time-induced deficit by Ro 04-6790 (King et al., 2004). These results support the theory that the cognitive enhancing effects observed following

treatment with 5-HT_6 receptor antagonists are sensitive to glutamatergic neurotransmission.

3.5 Key Findings

The findings from this chapter have highlighted the importance of selecting appropriate administration times when analysing the acute effect of systemic administration of SB-271046 in CER. CER relies upon the hippocampus, initial studies on 5-HT_6 receptor antagonists found an increase in acetylcholine and glutamate levels in the hippocampus and frontal cortex (Dawson et al., 2000, 2001; Riemer et al., 2003). The current data support this modulation of these neurotransmission systems, as post-training administration of SB-271046 partially reversed the scopolamine-induced memory impairment, and fully reversed the MK-801-induced deficit induced from scopolamine and MK-801. Together these studies support a role of 5-HT_6 antagonists in the treatment of cholinergic or glutamatergic cognitive deficits, which may aid the cognitive symptoms of disorders such as AD.

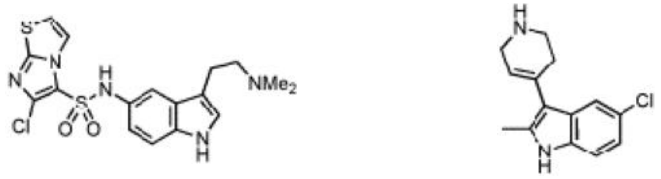
4 The effect of 5-HT$_6$ receptor agonists, EMD 386088 and E-6801, on conditioned emotion response

4.1 Introduction

The development of 5-HT$_6$ receptor agonists has been more difficult than the synthesis of antagonists, although, in the last decade, selective compounds have become available (Fone, 2008; Heal et al., 2008). Initial neurochemical studies led to the hypothesis that agonists acting on the 5-HT$_6$ receptor would impair learning and memory. One study analysed the pharmacological and neurochemical profile of two agonists, WAY 181187 and WAY 208466 using microdialysis (Schechter et al., 2008). WAY 181187 increased GABA levels within frontal cortex, striatum, hippocampus and amygdala, and decreased DA and 5-HT levels, these effects were blocked with pre-treatment of the antagonist, SB-271046, indicating the effects were mediated through 5-HT$_6$ receptors (Schechter et al., 2008). Furthermore, the effects of the agonists upon DA and 5-HT levels were attenuated with the GABA$_A$ receptor antagonist bicucilline, confirming an association between 5-HT$_6$ receptors and the GABA system (Schechter et al., 2008). Results from the study by Schechter and colleagues supported those of Woolley and colleagues (2004), that 5-HT$_6$ receptors are located upon GABA neurones. Within hippocampal slices, glutamate release was

stimulated via sodium azide and high potassium chloride, this was attenuated by WAY 181187 (Schechter et al., 2008). In contrast, findings from preliminary *in vivo* pre-clinical behavioural studies surprisingly found the selective 5-HT_6 receptor agonist *R*-13*c* (Cole et al., 2005b) reversed natural forgetting in the NOR paradigm analogous to the effect of 5-HT_6 receptor antagonists (Fone, 2008). Furthermore, the agonist WAY 181187 was recently found to facilitate the ED shift in the attention set shift learning and memory paradigm, (Burnham et al., 2010), although in the social recognition paradigm the same compound caused impairment (Loiseau et al., 2008). Therefore, much research is required to determine the effects of 5-HT_6 agonists upon learning and memory and to explain the mechanisms underlying the paradoxical similar effect of 5-HT_6 receptor agonists and antagonists on cognition.

No literature is available for the effects of 5-HT_6 receptor agonists on fear conditioning; therefore, the effects of two agonists, EMD 386088 and E-6801 (Figure 4-1), on CER were analysed in the current set of experiments. Previous *in vivo* and *in vitro* analysis has found EMD 386088 and E-6801 to be potent agonists at the 5-HT_6 receptor (Kendall et al., 2010; Mattsson et al., 2005; Romero et al., 2006).



(A) (B)

Figure 4-1-Chemical structures of (A) E-6801 and (B) EMD 386088

4.1.1 Aims

The aims of the studies in the current chapter were to assess the effects of acute administration of the 5-HT_6 receptor agonists, EMD 386088 and E-6801, on CER. This was achieved by:

- Determining the effects of 5-HT_6 receptor agonists on learning and memory in CER when administered alone.
- Analysing the effects of EMD 386088 and E-6801 on drug-induced cholinergic and glutamatergic deficits.

4.2 Methods

4.2.1 Animals

Adult male Lister Hooded rats (University of Nottingham BMSU, derived from Charles River stock, or Charles River UK) were housed in groups of 3-6. Rats were kept under the same standard laboratory conditions described in detail in Chapter 2 (Section 2.2.1).

4.2.2 Drugs

EMD 386088 (5-Chloro-2-methyl-3-(1,2,3,6-tetrahydropyridin-4yl)-1H -indole) was purchased from Tocris (Bristol, UK). E-6801 (6-chloro-N-(3-(2-(dimethylamino)ethyl)-1H-indol-5-yl)imidazo[2,1-b]thiazole-5-sulfonamide) was a gift from Esteve (Barcelona). Scopolamine hydrobromide and (+)-MK-801 maleate were purchased from Sigma Aldrich (Dorset, UK). EMD 386088 and E-6801 were dispersed in 0.154M saline containing 0.5% (w/v) methylcellulose,

and delivered in a volume of 3 ml kg^{-1}. Scopolamine and (+)-MK-801 were dissolved in sterile 0.154M saline and were delivered in 1 ml kg^{-1}.

4.2.3 Condition Emotion Response

The apparatus and behaviour training and testing protocols performed throughout this chapter follow those described in Chapter 2, section 2.2.3 and 2.2.4, with 3 administrations of the light, tone and foot shock at one minute intervals.

4.2.4 Experiment I: Effect of 5-HT_6 receptor agonists on memory consolidation in CER

The effects of either EMD 386088 or E-6801 were examined on memory consolidation using two separate groups of rats (n=7-9, weighing 260-325g and n=7-8, weighing 250-310g respectively). Rats in each group were randomly assigned into one of five treatments; vehicle + no shock; vehicle + shocked; EMD 386088 (10 mg kg^{-1}) or E-6801 (5 mg kg^{-1}) + no shock, EMD 386088 (5 and 10 mg kg^{-1}) or E-6801 (2.5 and 5 mg kg^{-1}) + shocked. Rats were trained in the CER paradigm (as previously described in Chapter 2), immediately following the conditioning trial rats received the relevant drug treatment. To determine any confounding effects of the 5-HT_6 receptor agonists which may have masked the freezing behaviour, the higher doses of EMD 386088 and E-6801 were also administered to rats that received no shocks. Following, training rats were returned to their home cage and tested 24 hours later.

4.2.5 Experiment II: Effect of 5-HT$_6$ receptor agonists on a scopolamine-induced cognitive deficit

As in previous chapters, scopolamine was utilised to induce a cholinergic memory deficit in CER and the ability of EMD 386088 and E-6801 to influence this impairment was determined using two separate groups of rats (n=7-9, weighing 245-310g and 240-305g accordingly). Rats in the two groups were randomly assigned into one of five treatment combination groups; saline + vehicle + no shock; saline + vehicle + shocked; scopolamine + vehicle + shocked; scopolamine + EMD 386088 (5mg kg^{-1}) or E-6801 (2.5 mg kg^{-1}) + shocked, and scopolamine + EMD 386088 (10mg kg^{-1}) or E-6801 (5 mg kg^{-1}) + shocked. Rats received the first injection, saline (0.3 ml kg^{-1}, i.p.) or scopolamine (0.3 mg kg^{-1}, i.p.) 20 minutes prior to training, the second injection, vehicle (0.5% methycellulose, 3 ml kg^{-1},i.p.), or EMD 386088 (5 or 10 mg kg^{-1}, i.p.) or E-6801 (2.5 or 5 mg kg^{-1}, i.p.) was administered immediately following training. In each experiment, one group of rats received scopolamine and vehicle in order to clarify the effects of scopolamine in this experiment. CER-induced freezing behaviour was measured 24 hours post-training by re-introduction to the test chamber without any further CS administration.

4.2.6 Experiment III: Effect of 5-HT$_6$ receptor agonists on a MK-801-induced cognitive deficit

Two groups of rats were randomly assigned into one of four treatment combination groups (n=6-8, weight=245-300g and n=8-9 weight=260-320g); saline + vehicle + no shock; saline + vehicle + shocked; MK-801 + vehicle +

shocked; and MK-801 + EMD 386088 + shocked, or MK-801 + E-6801 + shocked. To determine the effects of MK-801 on CER within each individual experiment, one group of rats received MK-801 and vehicle. Saline (1 ml kg^{-1}, i.p.) or MK-801 (0.1 mg kg^{-1}, i.p.) were administered 20 minutes prior to training, immediately following training rats were dosed with vehicle (0.5% methylcellulose, 3 ml kg^{-1}, i.p.) or EMD 386088 (5 mg kg^{-1}, i.p.) or E-6801 (2.5 mg kg^{-1}) accordingly.

4.2.7 Measured variables and Statistical Analyses

Latency time to cross into the dark chamber during CER training was automatically recorded by the ShutAvoid program. During the testing trial, the total cumulative time spent freezing was recorded manually by an observer blind to treatment.

Between-conditions ANOVA was utilised to determine any differences between drug treatments on latency times.

In order to maintain a robust parametric statistical analysis of the data, two separate analyses were performed to confirm that behaviour required the CS-US association and, separately, to examine the impact of 5-HT_6 receptor agonists on the CER response. To determine the difference between shock and no-shock-treated rats a Student's *t*-test was performed between the saline + vehicle + no shock and shock-treated groups, and the saline + agonist treatment no-shock and shock groups accordingly. Between-conditions analysis of drug treatment was

determined using one-way ANOVA with Tukey's post-hoc, on all shock treated groups.

4.3 Results

4.3.1 Experiment I: Effect of 5-HT$_6$ receptor agonists on memory consolidation in CER

To compare effects of 5-HT$_6$ receptor agonists with antagonists in CER, the agonists were administered immediately following training to determine the effects of activating 5-HT$_6$ receptors on CER. Effects of EMD 386088 will be discussed primarily in all experiments, on the training day, there was no difference in the latency to enter the dark chamber between groups in the study for EMD 386088 (Table 4-1A). This was as expected as no injection was given prior to training. To elucidate any confounding effects caused by EMD 386088 that may have masked the effects upon CER, the drug was administered to rats that received no foot shock. No differences were observed between this group and the vehicle + no shock group, with both groups freezing for less than 3% of the total test period, indicating EMD 386088 was having no unwanted effects in the paradigm. These results confirm the dependence of the CER on conditioning and that rats did not find the novel context aversive unless it had previously been paired with CS. Thus, freezing behaviour elicited by other groups was deduced to be due to an association between the context and shock. This study employed two doses of EMD 386088, 5 and 10mg kg^{-1}, to determine the effects of this compound on associative learning. All shock-treated rats, vehicle and both EMD 386088 groups, froze significantly longer than the no-shock-treated groups

(A)

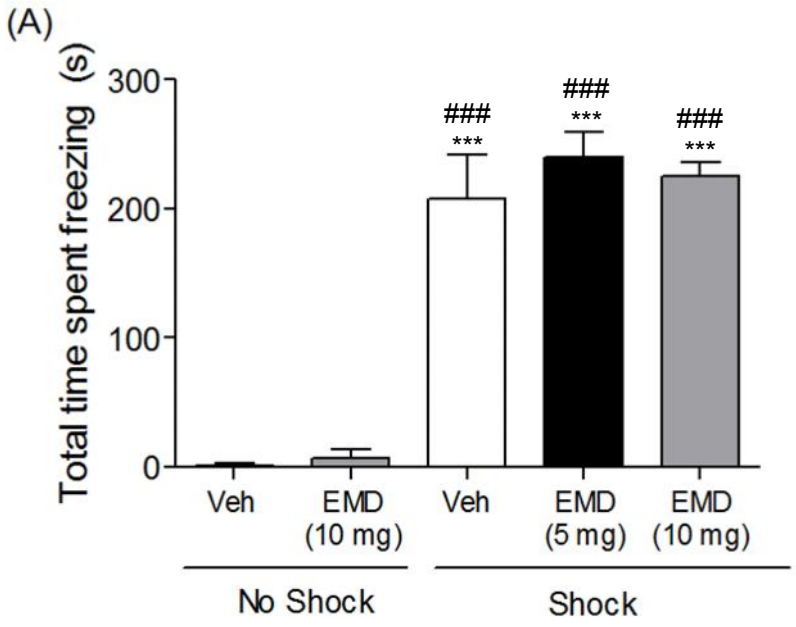
Treatment	Latency (sec)
Vehicle NS	12.8 ± 2.5
EMD 386088 (10mg kg^{-1}) NS	10 ± 2.3
Vehicle	11.3 ± 3.0
EMD 386088 (5mg kg^{-1})	9.4 ± 2.2
EMD 386088 (10mg kg^{-1})	7.5 ± 1.4

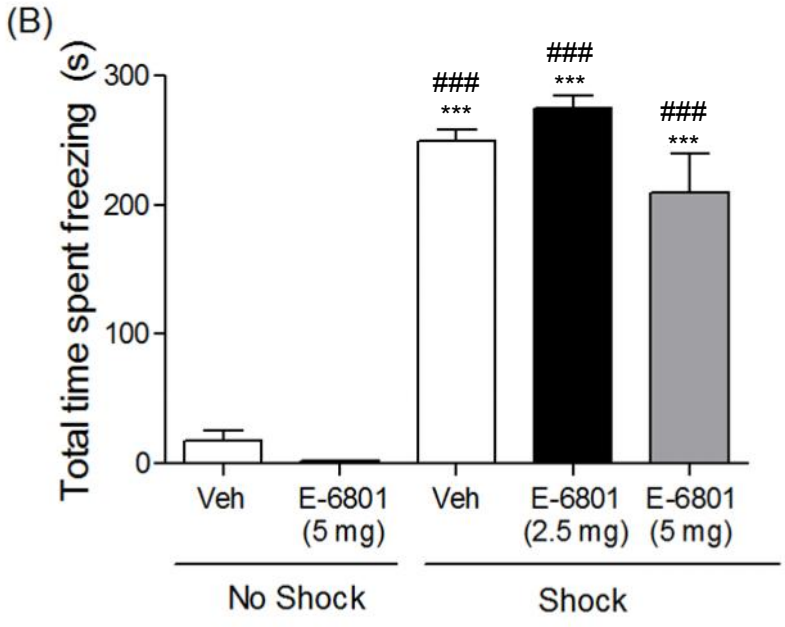
(B)

Treatment	Latency (sec)
Vehicle NS	10.7 ± 3.1
E-6801 (5mg kg^{-1}) NS	8.4 ± 1.7
Vehicle	9.3 ± 1.8
E-6801 (2.5mg kg^{-1})	11.6 ± 2.5
E-6801 (5mg kg^{-1})	12.6 ± 3.1

Table 4-1- Latency times (seconds, mean ± s.e.m) to cross into the dark chamber during CER training when effects of (A) EMD 386088 (n=7-9) or (B) E-6801 (n=7-8) were tested. No statistical differences were found between groups (ANOVA). NS = no shock.

Figure 4-2- No effects were observed when either (A) EMD 386088 or (B) E-6801 were administered immediately post-training on CER-induced freezing behaviour during the 24 hour retention trial. Total cumulative time spent freezing (seconds, mean ± s.e.m) during 24 hour retention trial in rats treated with (A) EMD 386088 (n=7-9) or (B) E-6801 (n=7-8). *** $p \leq 0.001$ versus vehicle + no-shock control group and ### $p \leq 0.001$ versus EMD 386088 (10mg kg^{-1}) or E-6801 (5 mg kg^{-1}) + no shock group accordingly (Student's *t*-test). No differences observed between shocked-treated groups (ANOVA). EMD = EMD 386088, veh = vehicle.


(A)
Total time spent freezing (s)
300
200
100
0
###
***
###
***
###
***
Veh
EMD (10 mg)
Veh
EMD (5 mg)
EMD (10 mg)
No Shock
Shock


(B)
Total time spent freezing (s)
300
200
100
0
###
***
###
***
###
***
Veh
E-6801 (5 mg)
Veh
E-6801 (2.5 mg)
E-6801 (5 mg)
No Shock
Shock

(Figure 4-2A, $p \leq 0.001$), with freezing times being over 69% of the total test period. No statistical differences were found between any shock-treated groups (Figure 4-2A, $p > 0.05$) implying that EMD 386088 alone had no effect on CER in normal adult rats.

Similar results were observed when testing the effects of E-6801 on CER-induced freezing behaviour. No differences were observed in latency times between groups (Table 4-1B, $p > 0.05$). E-6801 did not have any effects upon the subjects that could mask the behaviour elicited from CER, as no differences were found between no-shock-treated rats that received vehicle or E-6801 (Figure 4-2B, $p > 0.05$). The vehicle + no-shock group froze for 17.1 ± 8.2 seconds, appears to be longer than previous groups, which was due to three out of the seven subjects freezing over 30 seconds, but this is still less than 6% of the total test period (Figure 4-2B) and did not reach significance. E-6801 no shock treated rats froze for 1.6 ± 0.8 seconds, which is less than 1% of the total test period. Both of the no shock control groups indicate that the subjects did not find the novel environment aversive. All three shock treated groups froze significantly longer than the no-shock groups (Figure 4-2, $p \leq 0.001$), with each group freezing over 69% of the total test period. Although no statistical differences were observed between the three shocked-treated groups within the experiment it is interesting to note that the group which froze the longest received E-6801 at a dose of 2.5 mg kg^{-1} (274.9 ± 10 seconds), which is over 90% of the total testing period, the vehicle + shocked-treated rats froze for 249 ± 9.2 seconds slightly less than those treated with 2.5 mg kg^{-1} of E-6801 (Figure 4-2B).

Thus, both studies testing the effects of acute administration of 5-HT_6 receptor agonists post-training found these drugs to have no effect upon CER-induced freezing behaviour during the 24 hour retention trial.

4.3.2 Experiment II: Effect of 5-HT_6 receptor agonists on a scopolamine-induced cognitive deficit

These studies examined the ability of EMD 386088 and E-6801 to alter a cholinergic-induced memory deficit in CER produced by administration of scopolamine 20 minutes prior to training. There was no effect on the latency of rats to cross chambers during training were observed in the EMD 386088 study (Table 4-2A). As in previous experiments pre-training administration of the muscarinic antagonist, scopolamine, caused a significant reduction in freezing behaviour (86.4 ± 26.2 seconds) compared to the saline + vehicle shock treated group (223.2 ± 15.4 seconds) during the 24 hour retention trial (Figure 4-3A, $p \leq 0.01$). Two doses of EMD 386088 were used to test if activating the 5-HT_6 receptor could ameliorate the cholinergic deficit. The lower dose utilised (5mg kg^{-1}), tended to increase freezing behaviour (170.2 ± 31.4 seconds) compared to that in the scopolamine + vehicle treated group, although this just failed to reach significance ($p=0.08$, Tukey's post-hoc following ANOVA of shock treated groups). Interestingly the higher dose of EMD 386088, (10mg kg^{-1}), administered following pre-treatment of scopolamine significantly attenuated freezing behaviour (107.1 ± 17.5 seconds) compared to the saline + vehicle shock treated controls ($p \leq 0.01$). Within the scopolamine + EMD 386088 (10mg kg^{-1}) group, no subject froze for longer than 170 seconds, with four of the nine subjects

freezing for under 100 seconds. Rats that received no foot shock during CER training froze for 2% of the total test period, indicating that rats receiving shock froze due to an association between the context and aversive stimuli. Student's *t*-test performed to determine the difference between vehicle no shock and shock treated groups found that shock had a significant effect on CER-induced freezing behaviour (Figure 4-3A, $p \leq 0.001$).

An identical protocol was utilised to test the effects of E-6801 on a scopolamine-induced impairment in CER. This study found the 5-HT_6 receptor agonist, at 2.5 but not 5 mg kg^{-1}, reversed the cholinergic deficit in this cognitive paradigm (Figure 4-3B, $p \leq 0.05$). As with the previous experiments on pharmacological deficits rats received two injections, one prior to CER training and the second immediately following it. Latency times were automatically recorded and no statistical differences were found between groups that received saline and scopolamine prior to training (Table 4-2B), indicating that the primary injection had no adverse effects on behavioural analysis. Student's *t*-test showed that shocked-treated vehicle rats froze for significantly longer than no-shock vehicle controls ($p \leq 0.001$, Figure 4-3B), analogous to previous findings, further supporting the validity of the paradigm. Between-groups ANOVA ($F_{(3,33)}=9.363$, $p=0.001$), showed that E-6801 had a significant effect on CER-induced freezing. Post-hoc analysis showed scopolamine attenuated freezing behaviour (101.2 ± 17.2 seconds) compared to that of vehicle + vehicle shock treated rats (251.6 ± 11.7 seconds, $p \leq 0.001$) and this attenuation of freezing behaviour was significantly reversed by the lower dose of E-6801 (2.5 mg kg^{-1}, 181.9 ± 24.1 seconds, Figure 4-3B, $p \leq 0.05$). In contrast, the higher dose of E-6801 (5 mg kg^{-1})

(A)

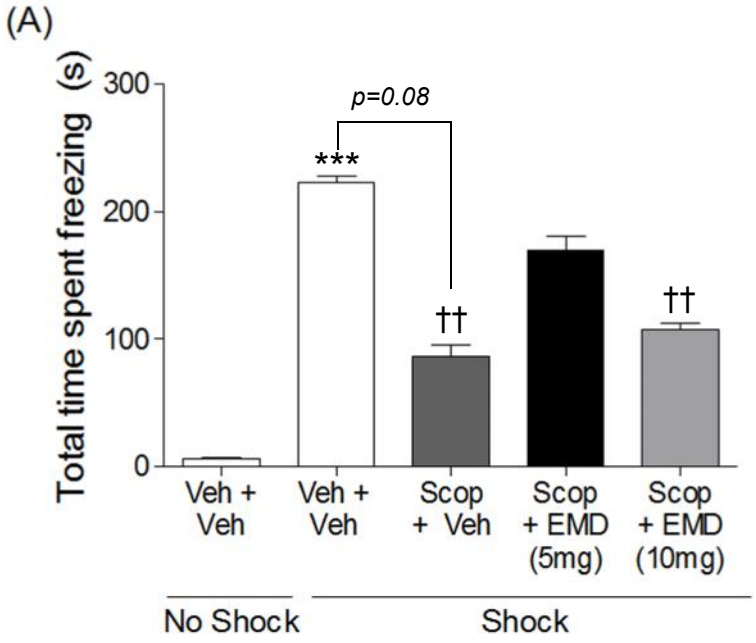
Treatment	Latency (sec)
Saline + Vehicle NS	14.3 ± 2.8
Saline + Vehicle	10.4 ± 1.6
Scop + Vehicle	6.9 ± 1.3
Scop + EMD 386088 (5mg kg^{-1})	9.2 ± 1.5
Scop + EMD 386088 (10mg kg^{-1})	8.4 ± 1.4

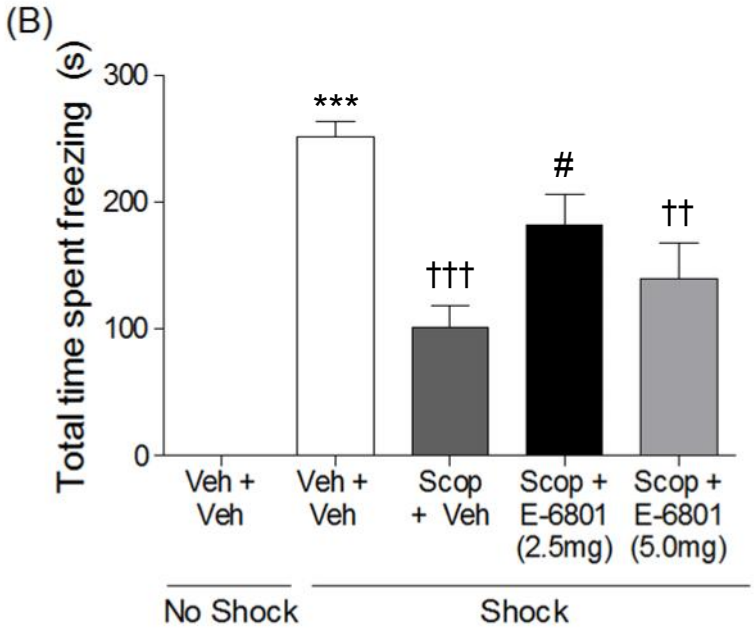
(B)

Treatment	Latency (sec)
Saline + Vehicle NS	10.8 ± 1.6
Saline + Vehicle	9.9 ± 2.3
Scop + Vehicle	7.0 ± 1.8
Scop + E-6801 (2.5 mg kg^{-1})	8.1 ± 1.3
Scop + E-6801 (5 mg kg^{-1})	6.9 ± 1.6

Table 4-2- Latency time (seconds, mean ± s.e.m) to cross from the light chamber into the dark chamber during CER training in experiments testing the effects of either (A) EMD 386088 (n=7-9) or (B) E-6801 (n=8-9) on scopolamine-induced memory deficit. No statistical differences were observed between groups (ANOVA). NS = no shock, scop = scopolamine.

Figure 4-3- Post-training administration of E-6801, reversed a pre-training scopolamine-induced memory deficit during 24 hour retention trial. Bar graph representing the total cumulative time spent freezing (seconds, mean ± s.e.m) in rats treated with either (A) EMD 386088 (5 and 10 mg kg^{-1}, n=7-9) or (B) E-6801 (2.5 and 5 mg kg^{-1}, n=8-9), following a cholinergic-induced memory impairment produced by administration of scopolamine 20 minutes prior to CER training trial. *** $p \leq 0.001$ versus saline + vehicle + no shock control group (Student's *t*-test), ††† $p \leq 0.001$ and †† $p \leq 0.01$ versus saline + vehicle shock group, # $p \leq 0.05$ versus scopolamine + vehicle shock group (Tukey's post-hoc following ANOVA of all shock treated groups). EMD = EMD 386088, scop= scopolamine, veh= vehicle.


(A)
Total time spent freezing (s)
300
200
100
0
p=0.08
***
††
††
Veh + Veh
Veh + Veh
Scop + Veh
Scop + EMD (5mg)
Scop + EMD (10mg)
No Shock
Shock


(B)
Total time spent freezing (s)
300
200
100
0
***
†††
#
††
Veh + Veh
Veh + Veh
Scop + Veh
Scop + E-6801 (2.5mg)
Scop + E-6801 (5.0mg)
No Shock
Shock

failed to significantly alter the attenuated freezing response by scopolamine such that the response was significantly less than that in the vehicle + vehicle shock treated rats ($p \leq 0.01$). This bell-shaped dose-response reversal is analogous to the effects of EMD 386088.

These two separate experiments have illustrated that 5-HT_6 receptor agonists, particularly E-6801 can exhibit reversal of a cholinergic impairment of CER (albeit a single dose).

4.3.3 Experiment III: Effect of 5-HT_6 receptor agonists on a MK-801-induced cognitive deficit

The effects of post-training administration of the 5-HT_6 receptor agonists on a glutamatergic-induced deficit in CER were tested next. EMD 386088 (5 mg kg^{-1}) administered post-training significantly reversed the attenuation of freezing behaviour induced from pre-training administration of the NMDA receptor antagonist MK-801 (Figure 4-4A, $p \leq 0.05$). During training MK-801 had no effect on the latency to enter the dark chamber (Table 4-3A). As expected shock administration had a significant effect upon freezing behaviour in rats re-exposed to the training context 24 hours post-training, as saline + vehicle + shock treated rats froze significantly longer (286.9 ± 5.9 seconds) than the no-shock control group (4.6 ± 2 seconds, Student's *t*-test). A significant main effect of drug treatment was confirmed with a one-way ANOVA ($F_{(2,21)}=5.794$, $p=0.011$) on freezing behaviour. Pre-training administration of MK-801 attenuated freezing behaviour (189.8 ± 33.5 seconds) compared to that of saline + vehicle + shocked

(A)

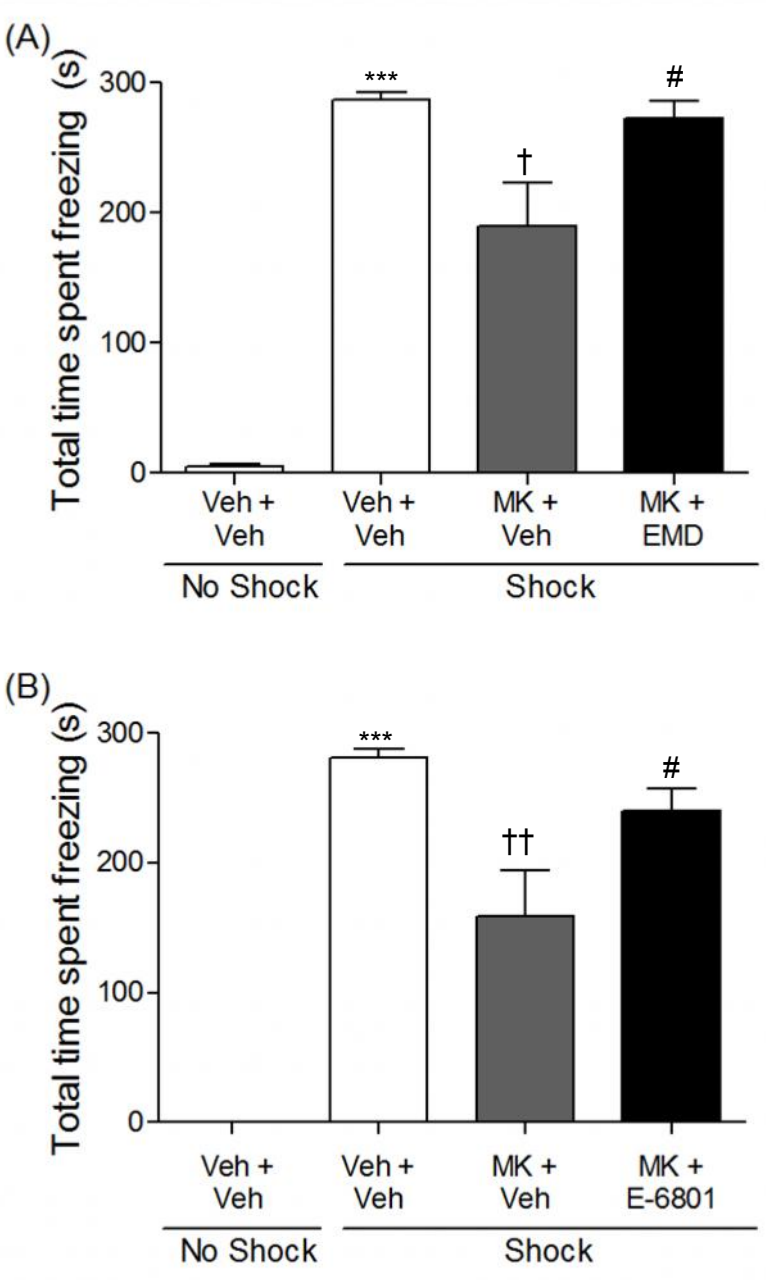
Treatment	Latency (sec)
Saline + Vehicle NS	10.1 ± 1.6
Saline + Vehicle	7.2 ± 1.0
MK-801 + Vehicle	7.7 ± 1.7
MK-801 + EMD 386088	11.5 ± 2.5

(B)

Treatment	Latency (sec)
Saline + Vehicle NS	9.6 ± 1.2
Saline + Vehicle	10.1 ± 1.8
MK-801 + Vehicle	20.8 ± 10.4
MK-801 + E6801	17.7 ± 4.8

Table 4-3- Latency time (seconds, mean ± s.e.m) to cross from the light chamber into the dark chamber during CER training in experiments testing the effects of either (A) EMD 386088 (5 mg kg^{-1}, n=6-8) or (B) E-6801 (2.5 mg kg^{-1}, n=8-9) on MK-801-induced memory deficit. No statistical differences were observed between groups (ANOVA). NS = no shock

Figure 4-4- Post-training administration of 5-HT_6 receptor agonists, EMD 386088 or E-6801, reversed pre-training MK-801-induced memory deficit during 24 hour retention trial. Bar graph representing the total cumulative time spent freezing (seconds, mean ± s.e.m) in rats treated with either (A) EMD 386088 (5 mg kg^{-1}, n=6-8) or (B) E-6801 (2.5 mg kg^{-1}, n=8-9) following a glutamatergic-induced memory impairment. *** *p≤0.001* versus saline + vehicle no-shock control group (Student's *t*-test), †† *p≤0.01* and † *p≤0.05* versus saline + vehicle shocked group, # *p≤0.05* versus MK-801 + vehicle shock group (Tukey's post-hoc following ANOVA). EMD = EMD 386088, MK = MK-801, veh = vehicle.


(A)
Total time spent freezing (s)
300
200
100
0
***
†
#
Veh + Veh
Veh + Veh
MK + Veh
MK + EMD
No Shock
Shock
(B)
Total time spent freezing (s)
300
200
100
0
***
††
#
Veh + Veh
Veh + Veh
MK + Veh
MK + E-6801
No Shock
Shock

treated rats (Figure 4-4A, $p \leq 0.05$, Tukey's post-hoc), which was fully reversed when EMD 386088 was administered following pre-treatment with MK-801 ($p \leq 0.05$). Thus, MK-801 + EMD 386088 + shock treated rats froze for 272.9 ± 13.8 seconds which was almost equivalent to the time spent freezing by the vehicle shocked-treated animals.

As with EMD 386088, post-training administration of E-6801 reversed the MK-801-induced impairment in CER, there being a significant effect of drug (ANOVA $F_{(2,24)}=7.923$, $p=0.003$) on freezing behaviour. Post-hoc analysis showed that shock treatment induced a freezing response 24 hours post-training, which was significantly ($p \leq 0.001$) greater than that seen in the saline + vehicle no-shock controls which did not freeze during testing period (Figure 4-4B). In contrast, rats given MK-801 prior to training elicited significantly less freezing behaviour (159.05 ± 35.09 seconds) than the saline + vehicle shock group (280.56 ± 7.15 seconds, $p \leq 0.01$, Figure 4-4B), showing that MK-801 induced a CER memory deficit. Of particular note, rats given E-6801 following pre-treatment with MK-801 froze for 240.08 ± 17.07 seconds, which is equivalent to the saline + vehicle shock controls showing that the 5-HT_6 receptor agonist reversed the glutamatergic-induced memory deficit ($p \leq 0.05$, Figure 4-4B).

Taken together these experiments show that two different 5-HT_6 receptor agonists can reverse both a cholinergic and a glutamatergic-induced deficit in a fear conditioning paradigm.

4.4 Discussion

The aim of the current chapter was to elucidate the effects of 5-HT_6 receptor agonists, EMD 386088 and E-6801, alone on CER, and then using pharmacological manipulation to determine the effects of these drugs on cholinergic and glutamatergic induced memory impairments.

As previously stated, initial reports proposed that 5-HT_6 receptor agonists would cause impairments in learning and memory paradigms (Fone, 2008). However, initial pre-clinical *in vivo* experiments performed to examine learning and memory changes proved controversial. WAY 181187 caused an impairment in the social recognition task in normal adult rats (Loiseau et al., 2008), but facilitated memory in the attentional set-shifting paradigm (Burnham et al., 2010). Only recently have selective 5-HT_6 receptor agonists suitable for *in vivo* studies become available and little literature exists on the two agonists utilised in the current chapter. One group found EMD 386088 to impair both short- and long-term memory in an associative autoshaping task (Meneses et al., 2008). A recent publication has analysed the effects of E-6801 on NOR in rats, when administered alone the agonist significantly increased the time spent exploring the novel object in a time-delay induced natural forgetting variant of the novel object recognition task, indicative of an enhancement of memory (Kendall et al., 2010). To date, there is much controversy regarding the effects of 5-HT_6 receptor agonists on learning and memory, and no literature is available on their effects on fear conditioning. In the previous study (Chapter 3) it was found that administration of the 5-HT_6 receptor antagonist, SB-271046, immediately following CER training was the optimal administration point. This protocol was

also used to examine the effect of the two selective high affinity 5-HT_6 receptor agonists, allowing for comparison of effects between two types of receptor ligands. The data obtained showed that neither EMD 386088 nor E-6801 given alone altered CER freezing. However, the freezing response is near maximal in this protocol so a ceiling effect may have prevented any prospect of observing an enhancement. In both experiments, the agonist-treated group, with the lower dose, froze for slightly longer than the vehicle + shock group, although this was not significant. The higher dose of E-6801, and to a lesser extent EMD 386088, froze less than the vehicle shock group, with the optimal dose appearing to be the lower one utilised (as also observed in the NOD paradigm by Kendall and colleagues 2010). It should be noted that neither EMD 386088 nor E-6801 caused an attenuation of freezing behaviour during the 24 hour retention trial, demonstrating that these two 5-HT_6 receptor agonists do not cause impairment of memory consolidation in CER.

As shown in Chapter 3, 24 hour retention of CER-induced memory can be impaired pharmacologically by pre-training treatment with drugs which attenuate either cholinergic or glutamatergic neurotransmission using the muscarinic receptor antagonist, scopolamine, or the NMDA receptor antagonist, MK-801. Both these compounds have been utilised to induced cognitive impairments in a variety of pre-clinical learning and memory tasks (Csernansky et al., 2005b; Lindner et al., 2006). Doses of scopolamine and MK-801 employed in the current chapter had no overt adverse behavioural effects which could have confounded data interpretation. There were no differences between-groups in the

latency exhibited to cross to the dark-side of the chamber during training in either study.

Pre-training administration of scopolamine impaired CER in both sets of agonist experiments. When either 5-HT_6 receptor agonist was administered immediately following training freezing behaviour increased close to that seen in vehicle treated controls, although this just failed to reach significance (*p=0.08*) with EMD 386088, E-6801 reversed the scopolamine-induced memory deficit. These results indicate that agonists at 5-HT_6 receptors restore memory that is impaired in a fear conditioning paradigm via modulation of cholinergic neurotransmission. Results from this study are comparable to those performed in our group on NOR (Kendall et al., 2010), where E-6801, at identical doses, reversed a scopolamine-induced deficit in NOR following a 1 minute inter-trial interval. Of note both agonists elicited a bell-shaped dose-response in the NOR (Kendall et al., 2010), and a similar pattern of results were observed in the present study, with higher doses of both EMD 386088 and E-6801 failing to reverse the scopolamine-induced cholinergic deficit in CER.

Previous studies found pre-training administration of MK-801 caused an impairment of memory in fear conditioning and NOR (Chapter 3, (Nilsson et al., 2007)). In the previous chapter SB-271046 was shown to fully reverse the glutamatergic impairment in CER induced through pre-training administration of MK-801. The current studies used an identical protocol to compare agonist and antagonist effects, both EMD 386088 and E-6801 fully reversed MK-801-induced memory impairments seen 24 hours post-training. As the agonists at the

5-HT_6 receptor reverse the behavioural effects observed following antagonism of the NMDA receptor it could be hypothesised that they increase glutamate neurotransmission. Neurochemical studies are required to confirm this hypothesis.

4.5 Key Findings

Findings from the current chapter provide strong evidence that 5-HT_6 receptor agonists, EMD 386088 and E-6801 had no effect upon CER when given alone, but both reversed impairments induced via modulation of both cholinergic and glutamatergic systems. Hence, the effects of 5-HT_6 receptor agonists on CER appear to be due to a modulation of both cholinergic and glutamatergic neurotransmission, and therefore could be beneficial in cognition. Administration of the 5-HT_6 receptor agonists, EMD 386088 and E-6801, immediately following CER training, allowed for comparison with SB-271046 findings. As previously stated CER relies upon the hippocampus, therefore the effects observed in the current series of studies are presumed to be due to neurochemical changes within this region. These results provide further evidence for the potential use of 5-HT_6 receptor ligands in the treatment of learning and memory deficits in AD and/or schizophrenia, but further research is required on the effects of 5-HT_6 receptor agonists. Due to the similar properties of 5-HT_6 receptor antagonists and agonists in CER the following chapter will attempt to find whether both compounds produced similar changes in protein expression within the hippocampus known to be involved in CER.

5 Effects of 5-HT$_6$ receptor ligands on hippocampal protein expression

5.1 Introduction

Literature regarding protein synthesis and memory formation provides strong evidence that the former is required, either during or shortly after training to form a long-term memory (LTM) (Davis and Squire, 1984). One group utilised protein synthesis inhibitors to show that protein synthesis is required for LTM formation; mice learnt a task with inhibition of protein synthesis and could remember up to 3 hours post-training but could not form a LTM (Barondes and Cohen, 1968). Following a fear-motivated learning and memory task, NMDA receptors are activated together with downstream molecular pathways, and changes in expression of transcription factors, those known to be involved in this process are discussed in Chapter 2 (Izquierdo and Medina, 1997). This is very similar to the process of LTP (Bliss and Collingridge, 1993), a candidate process of memory consolidation. One downstream protein known to be activated in LTP is brain derived neurotrophic factor (BDNF), hence the expression of this, and the 5-HT$_6$ receptor itself are analysed in the current chapter.

5.1.1 Aims

The aim of the current chapter was to determine any differences in hippocampal protein expression of BDNF and 5-HT_6 receptor following treatment with 5-HT_6 receptor ligands.

5.2 Materials and Methods

5.2.1 Samples

All hippocampus samples were taken 1 hour post CER testing from previous behavioural experiments (Chapters 3 and 4). Rats were killed (using a Schedule 1 procedure) and whole hippocampi were dissected, weighed and frozen immediately in liquid nitrogen, prior to storage at -80°C. Experimental tissue was taken from following experiments:

- SB-271046 on scopolamine-induced deficit (n=6-9)
- SB-271046 on MK-801-induced deficit (n=8)
- EMD 386088 on scopolamine-induced deficit (n=8-9)
- EMD 386088 on MK-801-induced deficit (n=6-8)

For each individual experiment BDNF and 5-HT_6 receptor protein levels were measure using Western blotting.

5.2.2 Western blotting

Western blotting utilises SDS-PAGE to separate protein mixtures; sample homogenates are denatured and separated according to their molecular weight.

Proteins are transferred to a nitrocellulose membrane and a specific antigen is detected using an antibody.

5.2.2.1 Sample Preparation

Hippocampus samples were homogenised (samples kept on ice, 1 minute, ULTRA-TURRAX homogeniser, T-10 Basic, IKA) in lysis buffer (20 mM Tris, 1mM EGTA, 320mM sucrose, 0.1% Triton X100, 1mM NaF, 10mM beta glycerophosphate, dissolved in 500ml dH_20 containing protease inhibitors, pH 7.6), to make a tissue concentration of 100 mg ml^{-1}. Samples were centrifuged (Sigma 3-18K) for 5 minutes, 13 000 rpm (15493 g) at 4°C, prior to removing supernatant. To determine protein concentration a Lowry protein assay (Lowry et al., 1951) was performed, each sample was then normalised to the lowest protein concentration by adding appropriate volumes of solubilisation buffer (0.125M Tris, 20 % glycerol (v/v), 2% sodium dodecyl sulphate (SDS, v/v), 10% β-mercaptoethanol (v/v), 0.01% bromophenol blue (w/v) and dH_2O) to each sample. This ensured that any differences observed were due to treatment rather than concentration differences.

5.2.2.2 Gel electrophoresis

SDS-PAGE gels were prepared (Acrylamide (Protogel), Tris (0.5M resolving and 1.5M stacking), 10% SDS, 10% ammonium persulfate (APS), (N,N,N′,N′-tetramethylethylenediamine (TEMED)) for each individual experiment:

- BDNF- utilised 12% acrylamide gels.
- 5-HT_6 receptor- utilised 10% acrylamide gels.

Gels were loaded with 10 μg of protein per well, with a molecular weight marker (10-260 kDa, Fermentas or 10-250 kDa, Bio-Rad) and a positive control for the specific protein of interest. Proteins (first heated at 92°C for 5 minutes) were separated via electrophoresis (electrode buffer: 0.025M Tris containing 0.19M glycine, 0.001% (v/v) SDS):

- BDNF- performed at 200V for approximately 45 minutes.
- 5-HT_6 receptor- performed at 62V, 30 mA for 20 minutes, then 155V, 60mA for 60 minutes.

5.2.2.3 Protein transfer

On completion of electrophoresis, proteins were transferred from gels onto a nitrocellulose membrane (Hybond, ECL), in transfer buffer (0.025M tris containing 0.19 M glycine, 20% (v/v methanol) in the cold room (4°C):

- BDNF- 100V for 60 minutes.
- 5-HT_6 receptor- 45V for 90 minutes.

To confirm the transfer of proteins the nitrocellulose membrane was reversibly stained with Ponceau S solution (Sigma, UK), the position of the molecular weight markers was identified and marked. The membrane was then de-stained in TBST (25mM Tris, 125mM NaCl, 10 L dH_2O, 10 ml Tween 20, pH 7.6).

5.2.2.4 Blocking

Non-specific binding of proteins was prevented by blocking the nitrocellulose prior to the addition of antibodies:

- BDNF- Snap ID cassettes (Millipore) were soaked in dH_20 prior to use. Nitrocellulose was blocked with fish skin gelatine (Sigma, UK, 1:30 dilution with TBST), poured directly into the cassette and pumped through.
- 5-HT_6 receptor- The nitrocellulose membrane was blocked in 5% (w/v) milk powder (1 g milk powder in 20 ml TBST) for 30 minutes at room temperature on the rocking platform.

5.2.2.5 Antibodies

Nitrocellulose membranes were incubated with relevant antibodies:

- BDNF- rabbit polyclonal IgG BDNF (N-20, Santa Cruz, 1:500) in fish skin gelatine (1:30), membrane incubated for 10 minutes. The primary antibody was then removed and the nitrocellulose membrane was washed three times with TBST. The membrane was incubated in fish skin gelatine (1:30) containing secondary antibody, IRDye 800CW goat anti-rabbit (Li-Cor, 1:2500), for 10 minutes (darkened room due to light sensitive antibody). The membrane was washed three times with TBST and placed in dH_2O prior to scanning.

- 5-HT_6 receptor- A polyclonal antibody previously raised in sheep against the N-terminal portion (Pro^3-Trp^{15}) of the rat 5-HT_6 receptor (anti-3-

15YC) (Woolley 2002; Woolley et al 2004), this was utilised throughout the current set of studies, designed due to its limited homology with other proteins (BLAST database search). The nitrocellulose membrane was incubated with anti-3-15YC antiserum (1:500) in blocking buffer for 1 hour at room temperature. The membrane was washed three times with TBST prior to incubation with the secondary antibody (rabbit anti-sheep, Dako, 1:5000) in blocking buffer for 30 minutes at room temperature. The nitrocellulose membrane was washed in TBST and kept in dH_20.

5.2.2.6 Developing the film

- BDNF- Nitrocellulose membranes were scanned using an Odyssey Infrared Imaging System Scanner (Li-Cor, Cambridge, UK), the molecular weight and optical densities of resulting bands were analysed with Odyssey v3 software package.
- 5-HT_6 receptor- To visualise immunoreactive proteins the enhanced chemiluminescence (ECL) procedure was performed, nitrocellulose membranes were incubated in 1:1 ECL (3ml/blot, GE Healthcare) for 3 minutes, and then exposed to blue X-ray film (PIERCE). Molecular weight and optical densities of the bands were analysed in the ImageJ One software package.

5.2.3 Measured variables and Statistical Analyses

As mentioned above optical densities of protein bands were determined using specific software packages, Odyssey and ImageJ. The house-keeping protein for

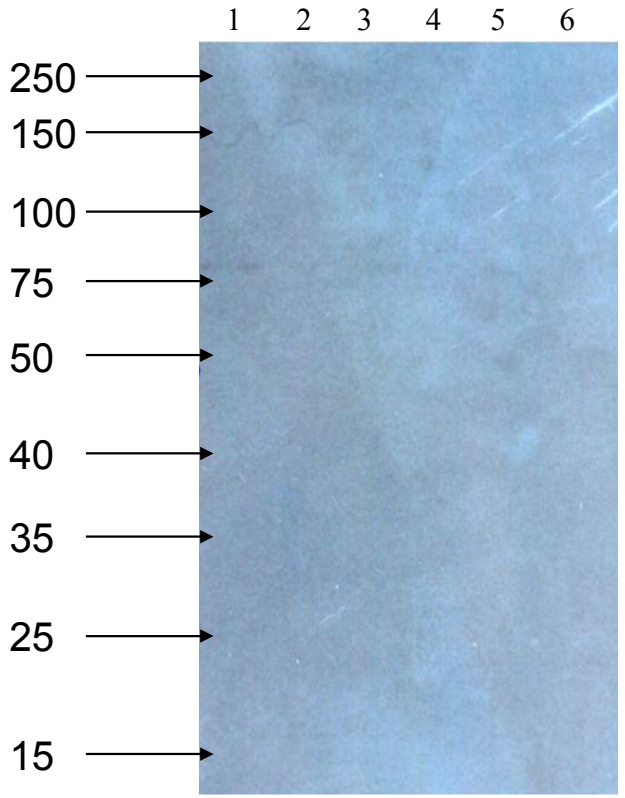
all experiments was GAPDH. The protein of interest for each lane within an experiment was normalised to the GAPDH signal for that lane to eliminate differences due to any loading discrepancies.

Two separate statistical tests were performed to maintain a parametric analysis system. To determine the effect of shock on protein expression a Student's *t*-test was performed between the saline + vehicle + no-shock and shock treated groups. Between-conditions analysis of drug treatment upon expression levels of proteins was determined using one-way ANOVA with Tukey's post-hoc, on all shock treated groups.

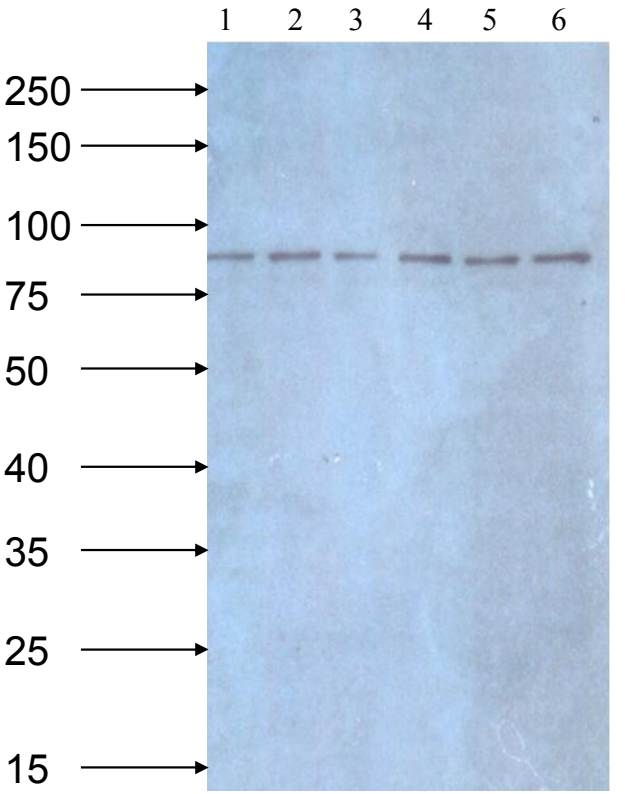
5.3 Results

Prior to studies with experimental tissue preliminary studies were performed to optimise the techniques for the individual protein detection, these utilised hippocampal tissue taken from a CER behavioural experiment (behavioural data not shown), and which were, therefore, were representative of experimental tissue. Unlike the 5-HT_6 receptor antibody, the BDNF primary antibody was commercially available (Santa Cruz) and therefore, limited optimisation was required. Serial dilution studies found that 1:500 was optimal for the primary antibody dilution in detecting BDNF protein expression in hippocampal tissue, and Li-Cor recommended 1:2500 for the secondary antibody. Studies found a strong band at ~26 kDa, a weaker band was observed at 13kDa (Figure 5-2A). The molecular weight of BDNF is 13 kDa, therefore the band observed at 26 kDa is the homodimer of the neurotrophin protein.

Figure 5-1- Specificity of the 5-HT_6 receptor protein antibody was provided with immunoblot analysis of hippocampal tissue probed with (A) pre-immune serum or (B) purified anti-3-15YC at 1:500 dilution. Incubation with the purified anti-3-15YC serum highlighted a band at ~90kDa, which was absent when membrane was incubated with the pre-immune serum. Image is representative of experimental tissue, hippocampal samples were collected 24 hours following CER training, lanes 1 and 5 were no shock treated rats, lanes 2, 4 and 6 were shock treated rats and lane 3 was a hippocampus from a naïve rat, i.e. had remained in the home cage throughout behavioural experiment. Numbers alongside each blot represent the molecular weight (kDa) of the proteins, and are estimated from molecular protein marker (10-250 kDa, Bio-Rad).

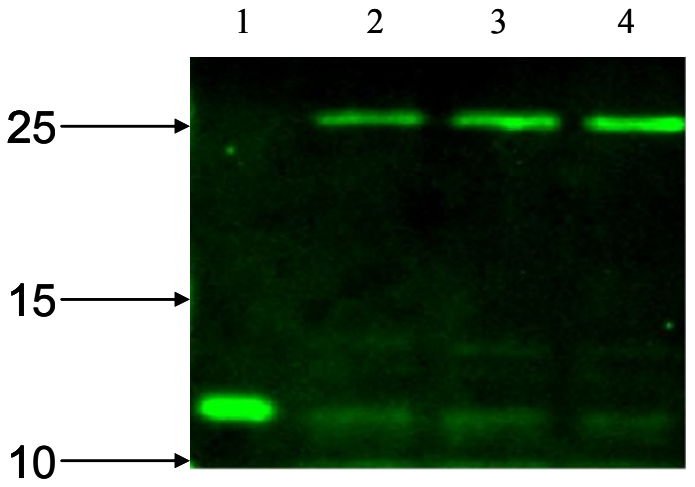

1 2 3 4 5 6
250
150
100
75
50
40
35
25
15

(A)

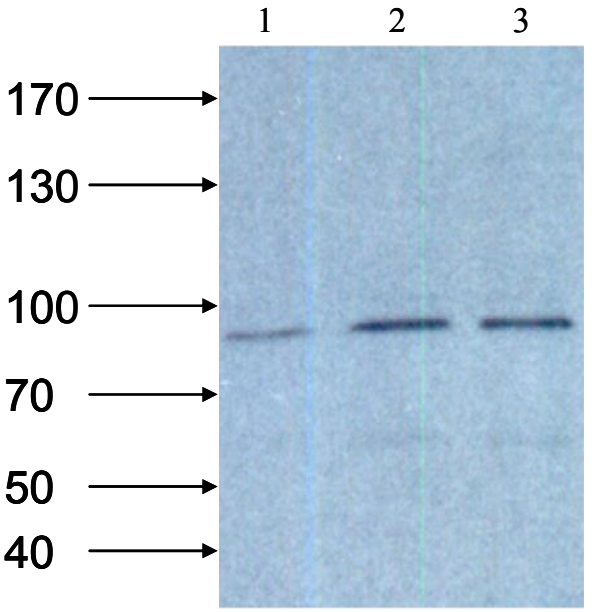

1 2 3 4 5 6
250
150
100
75
50
40
35
25
15

(B)

Figure 5-2- Two different visualisation techniques were utilised to determine the hippocampal expression of (A) BDNF and (B) 5-HT_6 receptor levels. To analyse expression of BDNF Odyssey was utilised which detects protein bands using a direct infrared fluorescence system, 5-HT_6 receptor expression was detected using a chemiluminescence technique. Numbers alongside each blot represent the molecular weight (kDa) and are estimated from molecular protein marker (10-260 kDa, Fermentas). Image is representative of experimental tissue, as hippocampi samples utilised in current experiment were collected 24 hours following CER training. In Figure A, lane 1 = recombinant BDNF, lane 2 = no shock treatment, lane 3 = shock treatment, and lane 4 = naïve tissue. For figure B, lane 1 = no shock treatment, lane 2 = shock treatment, and lane 3 = naïve tissue, i.e. had remained in the home cage throughout behavioural experiment.



(A)



(B)

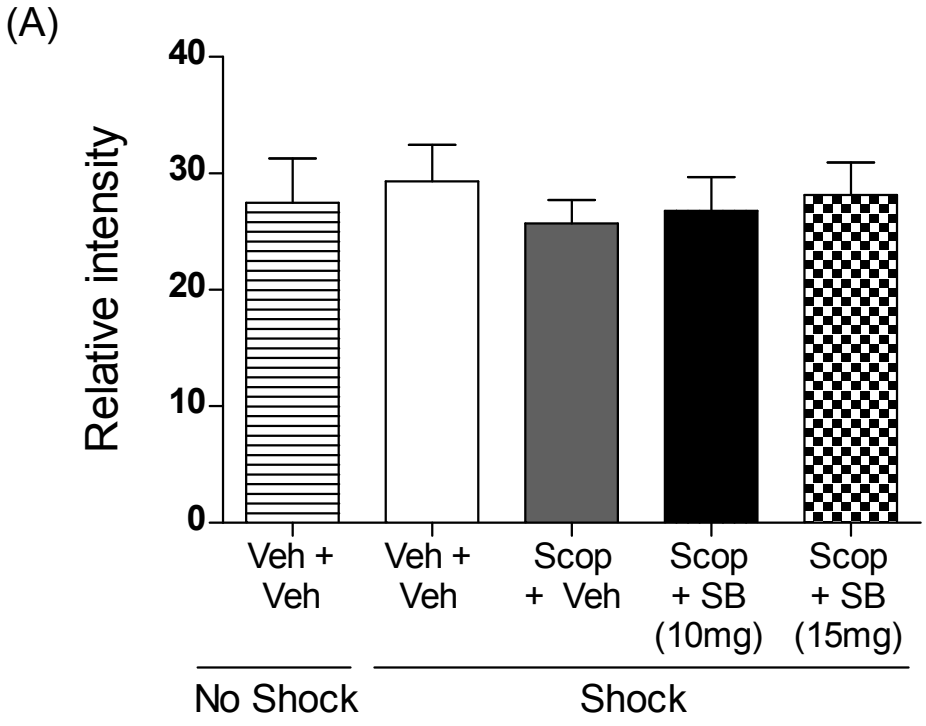
Optimisation studies for 5-HT_6 receptor found following incubation with anti-3-15YC antibody there was a strong band at 90 kDa (Figure 5-1B, Figure 5-2B), which was not observed following incubation with pre-immune serum (Error! Reference source not found.A), suggesting that this band is not due to non-specific binding but is a homodimer of the 5-HT_6 receptor protein. The molecular weight of the 5-HT_6 receptor protein is 46 kDa (Kohen et al 1996), and due to the cysteine residues present in the receptor it is possible for the protein to exist as a dimer. Optimisation utilised serial dilutions of the purified antibody to determine the optimal concentration to visualise protein bands, it found that 1:500 (data not shown) was the optimal dilution of primary antibody.

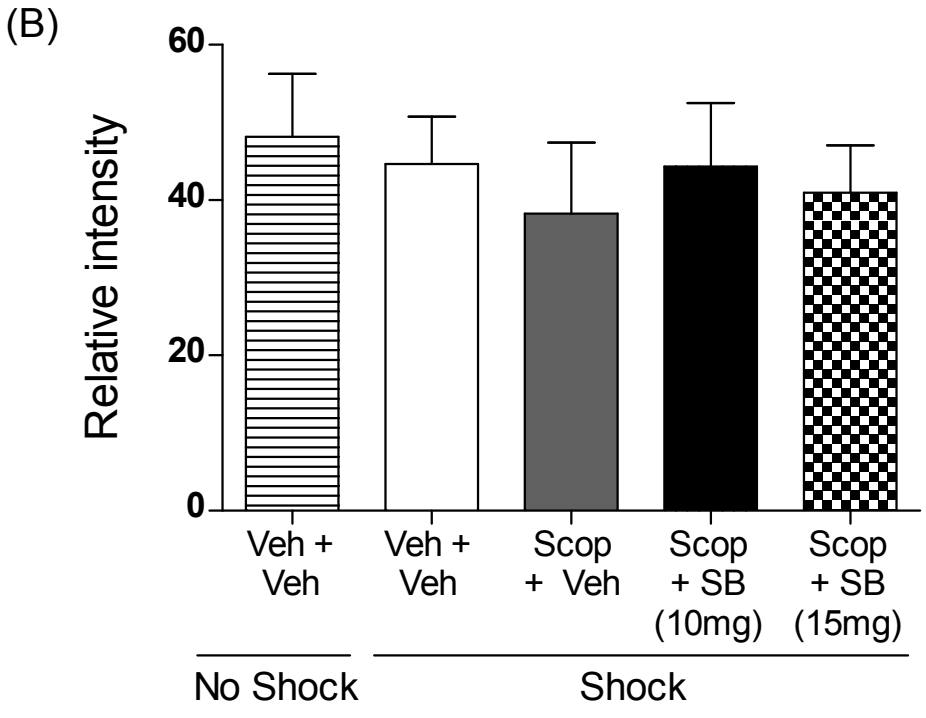
The current set of studies prepared the tissue homogenates in solutions containing β-mercaptoethanol, this only removes disulphides and dimers are held together with hydrophobic forces, hence the strong bands at the molecular weight of the dimers observed in the current studies must be SDS stable.

5.3.1 Experiment I- SB-271046 and Scopolamine 24 hours post-training

No statistical differences were observed between drug + shock-treated groups (ANOVA, $F_{(3,29)}$=0.296, *p=0.828*) when analysing the expression of BDNF protein levels in SB-271046 treated rats on a cholinergic-induced deficit (Figure 5-3A). Student's *t*-test found no difference between vehicle-treated rats that received either foot shock or were controls.

Figure 5-3- Western blot analysis of hippocampal (A) BDNF, and (B) 5-HT_6 receptor, protein expression 24 hours following the behavioural study analysing the effects of SB-271046 on scopolamine-induced memory impairment found no statistical difference between groups. Bar graph representing the average optical density/relative intensity of protein (mean ± s.e.m, n=6-9), following normalisation to GAPDH. No statistical differences were observed for shock or drug treatment on either protein. Hippocampal tissue taken from behavioural Experiment II described in Chapter 3, section 3.2.5. SB = SB-271046, scop = scopolamine and veh = vehicle.


(A)
Relative intensity
40
30
20
10
0
Veh + Veh
Veh + Veh
Scop + Veh
Scop + SB (10mg)
Scop + SB (15mg)
No Shock
Shock


(B)
Relative intensity
60
40
20
0
Veh + Veh
Veh + Veh
Scop + Veh
Scop + SB (10mg)
Scop + SB (15mg)
No Shock
Shock

In the same experimental group, shock treatment did not cause any change in expression levels of 5-HT_6 receptor protein (Figure 5-3B). As with BDNF expression no significance was observed between treatment groups with ANOVA. Interestingly the lower dose of SB-271046 increased 5-HT_6 receptor expression, with the higher dose eliciting similar levels to that of the scopolamine-treated group (Figure 5-3B).

5.3.2 Experiment II- SB-271046 and MK-801

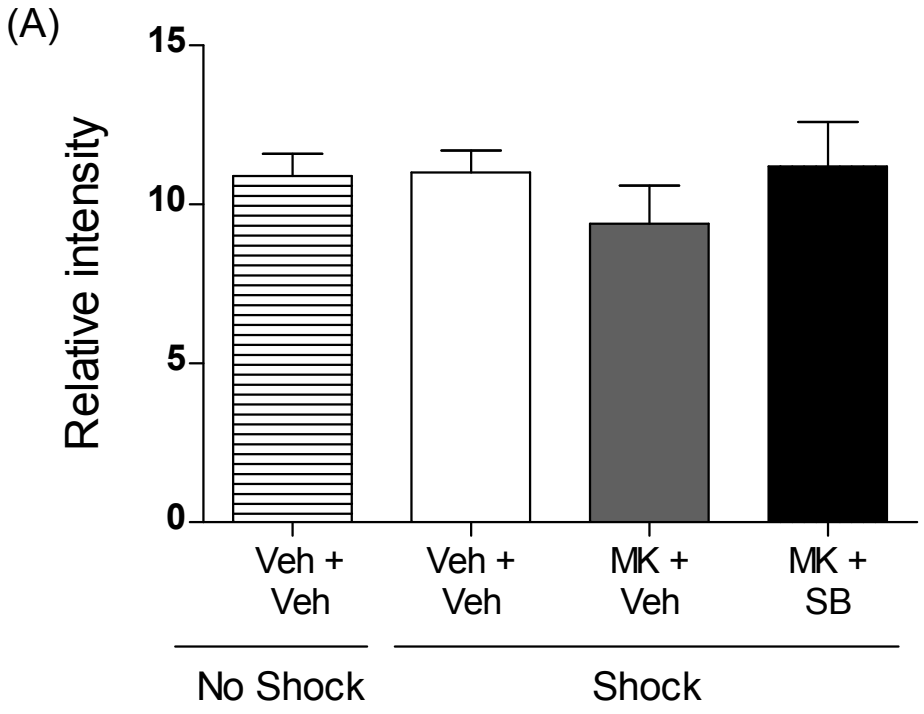
No statistical difference was observed between shock (Student's *t*-test, *p=0.9210*) or treatment (ANOVA, $F_{(2,23)}$=0.728, *p=0.495*) groups in analysis of BDNF expression levels following SB-271046 on MK-801-induced memory deficits.

Expression levels of hippocampal 5-HT_6 receptor protein in SB-271046 on MK-801-induced deficit groups were very similar (66.7 ± 7.8 - 82.7 ± 16.4 optical density). Neither shock nor drug treatment had any statistical effect on the levels of 5-HT_6 receptor protein (Student's *t*-test *p=0.8948,* ANOVA $F_{(2,20)}$=0.453, *p=0.643*) (Figure 5-4B).

5.3.3 Experiment IV- EMD 386088 and Scopolamine

Although there were slight differences between groups no significant effect of drug + shock treatment were observed (Figure 5-5A). It should be noted that there are large standard errors within this experiment due to high background noise on one of the membranes, no sample was emitted due to this to allow a direct comparison of any molecular findings to behavioural results (Chapter 4).

Figure 5-4- Western blot analysis of hippocampal (A) BDNF, or (B) 5-HT_6 receptor, protein expression 24 hours following the behavioural study analysing the effects of SB-271046 on an MK-801 induced memory deficit found no statistical difference between groups. Bar graph representing the average optical density/relative intensity of protein (mean ± s.e.m, n=8), following normalisation to GAPDH. No statistical significance was observed between shock or drug treatments for either BDNF or 5-HT_6 receptor protein expression. Hippocampus samples were taken immediately following behavioural Experiment III described in Chapter 3, section 3.2.6. MK = MK-801, SB = SB-271046 and veh = vehicle.


(A)
Relative intensity
15
10
5
0
Veh + Veh
Veh + Veh
MK + Veh
MK + SB
No Shock
Shock

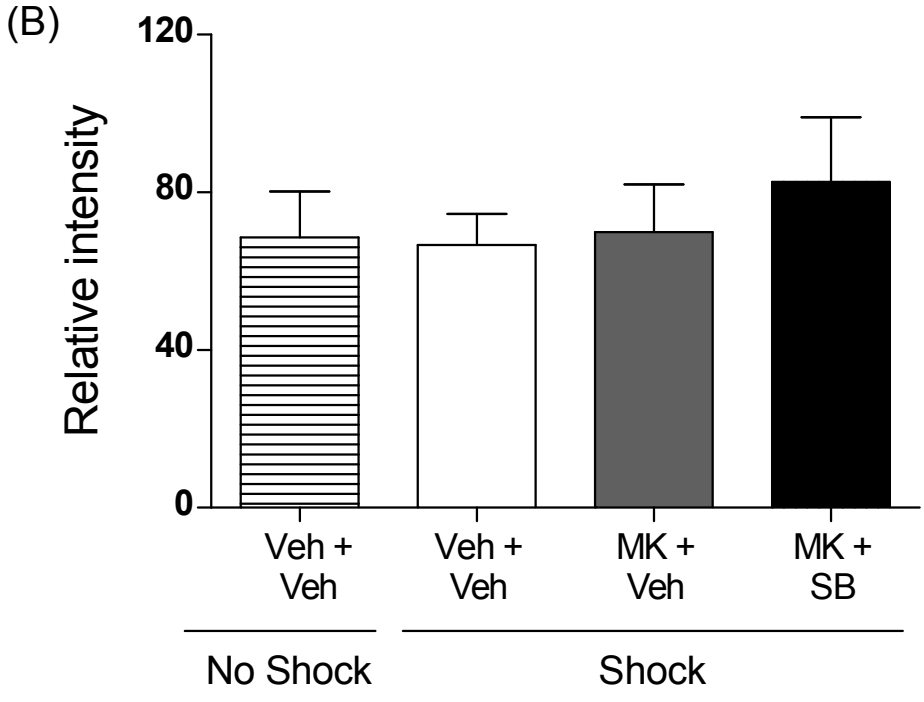
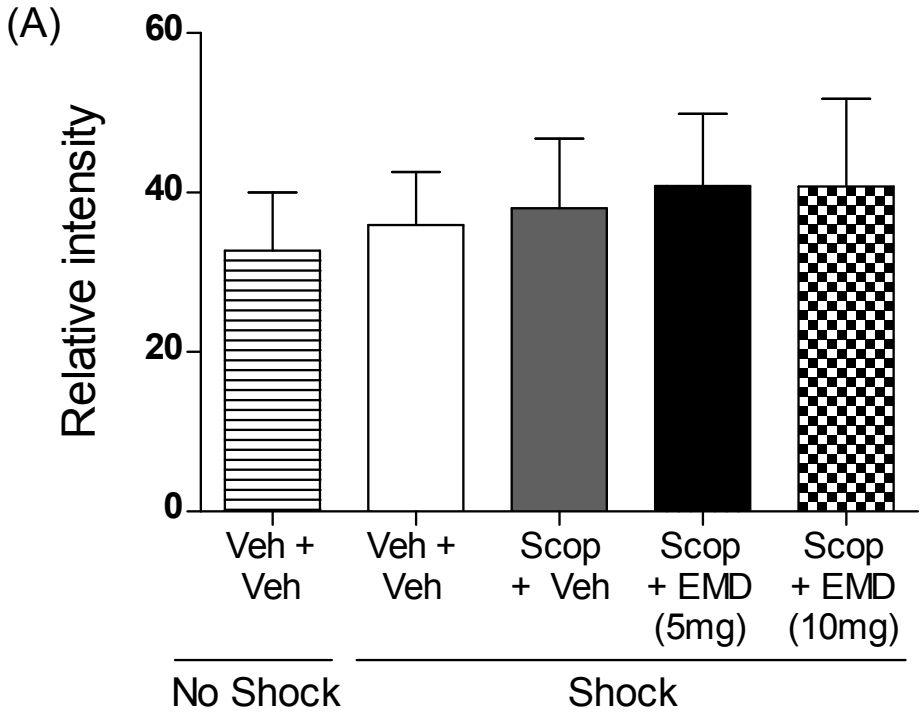

(B)
Relative intensity
120
80
40
0
Veh + Veh
Veh + Veh
MK + Veh
MK + SB
No Shock
Shock

Figure 5-5- Western blot analysis of hippocampal (A) BDNF, or (B) 5-HT_6 receptor, protein expression 24 hours following the behavioural study analysing the effects of EMD 386088 on a scopolamine induced memory deficit in CER found no statistical difference between groups. Bar graph representing the average optical density/relative intensity of protein (mean ± s.e.m, n=8-9), following normalisation to GAPDH. No statistical differences were observed between groups for either protein. Hippocampal samples were taken following behavioural Experiment II, described in Chapter 4, section 4.2.5. EMD = EMD 386088, scop = scopolamine, veh = vehicle.


(A)
Relative intensity
60
40
20
0
Veh + Veh
Veh + Veh
Scop + Veh
Scop + EMD (5mg)
Scop + EMD (10mg)
No Shock
Shock

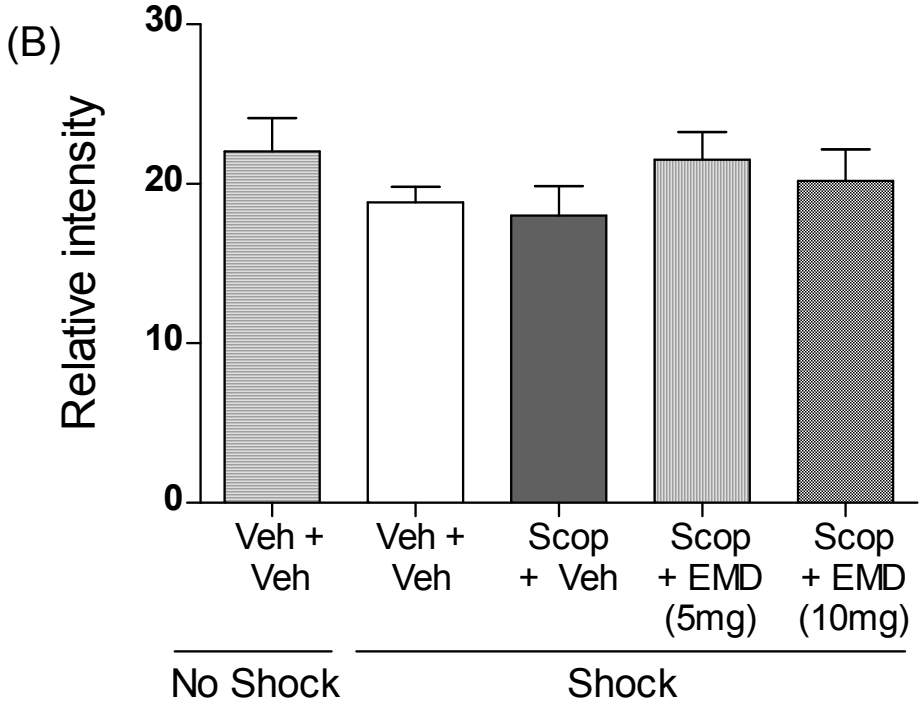
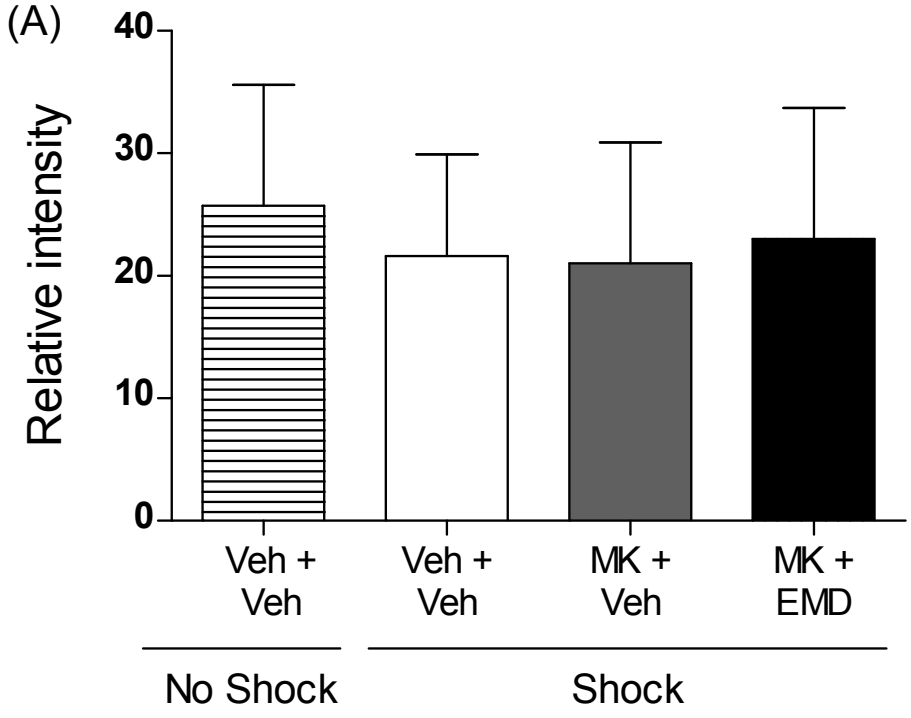
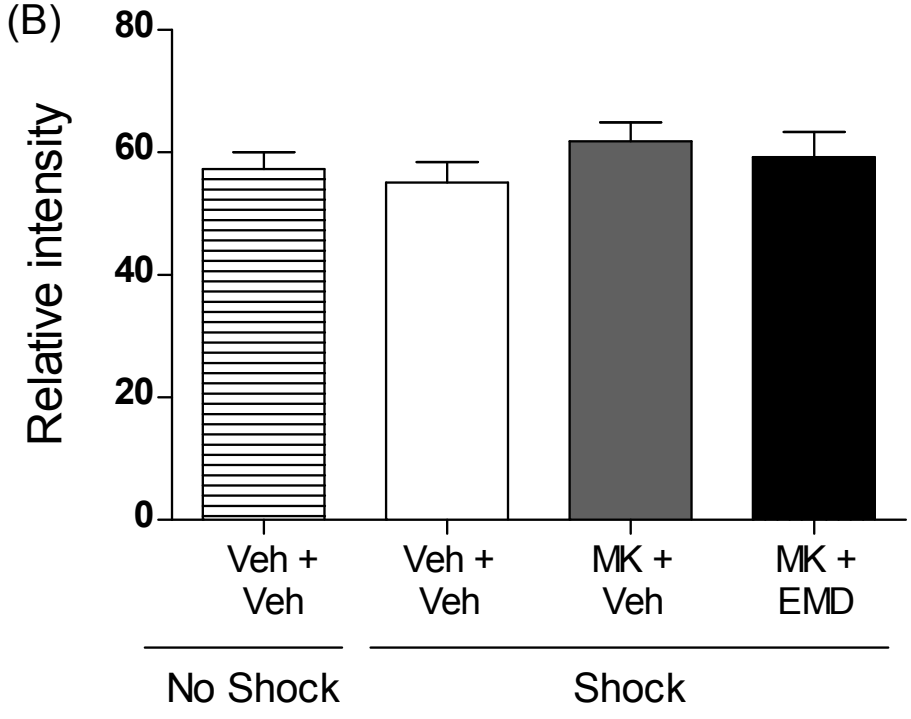

(B)
Relative intensity
30
20
10
0
Veh + Veh
Veh + Veh
Scop + Veh
Scop + EMD (5mg)
Scop + EMD (10mg)
No Shock
Shock

Figure 5-6- Western blot analysis of hippocampal (A) BDNF, or (B) 5-HT_6 receptor, protein expression 24 hours following the behavioural study analysing the effects of EMD 386088 on an MK-801 induced memory deficit found no statistical difference between groups. Bar graph representing the average optical density/relative intensity of protein (mean ± sem, n=6-8), following normalisation to GAPDH. No differences were observed between any treatment groups. Hippocampal samples were collected following behavioural Experiment III, described in Chapter 4, section 4.2.6. EMD = EMD 386088, MK = MK-801, veh = vehicle.


(A)
Relative intensity
40
30
20
10
0
Veh + Veh
Veh + Veh
MK + Veh
MK + EMD
No Shock
Shock


(B)
Relative intensity
80
60
40
20
0
Veh + Veh
Veh + Veh
MK + Veh
MK + EMD
No Shock
Shock

The expression level of BDNF protein failed to show a significant difference in shock and no shock treated groups (Student's *t*-test *p=0.7516*).

A Student's *t*-test found no significance between the saline + vehicle shock and no-shock treatment groups (*p=0.16*) (Figure 5-5B). Drug treatment had no effect on the expression levels of hippocampal 5-HT_6 receptor protein (*p>0.05*).

5.3.4 Experiment V- EMD 386088 and MK-801

The signal from the housekeeping protein, GAPDH, was low on one of the membranes analysed in the current study, hence increasing the standard error of the overall data. Although no significance was observed there was a similar effect to that observed with SB-271046 on an MK-801 induced deficit (Figure 5-6A). Neither shock nor drug treatment had an effect on the expression levels of hippocampal BDNF in this experiment.

None of the drug + shock treatment groups had any effect upon hippocampal levels of 5-HT_6 receptor protein expression ($F_{(2,20)}$=1.013, *p=0.383*). The shock treatment had no effect on the 5-HT_6 receptor expression levels (Student's t-test, *p>0.05*, Figure 5-6B).

5.4 Discussion

The main aim of this chapter was to determine any differences in hippocampal BDNF and 5-HT_6 receptor protein expression levels, using Western blot protocols. As little is known regarding the underlying molecular pathways linked

to the 5-HT_6 receptor, examining the effects of the antagonist and agonist alone and, following a cholinergic- or glutamatergic-induced deficit in learning and memory were of great interest.

BDNF is a neurotrophin, the expression of which is required for synaptic plasticity and hippocampal-dependent memory formation (Korte et al., 1995; Mizuno et al., 2000). Due to this, several groups have analysed the expression of this protein within the hippocampus following various pre-clinical behavioural paradigms, including contextual fear conditioning (Chapter 2, section 2.1.3) (Barrientos et al., 2004; Hall et al., 2000; Mizuno et al., 2000). In the current set of studies no significant differences between any treatment combination groups were observed. This may have been due to the hippocampal samples for our analyses being taken 24 hours following training, after expression changes may have returned to baseline. Other groups showed an increase in BDNF mRNA levels at 0.5 and 2 hours post-training, although no differences were observed between unconditioned and conditioned subjects at 4 and 6 hours post-training (Barrientos et al., 2004; Hall et al., 2000; Rattiner et al., 2004). In contrast, another group found an increase in BDNF levels, in both young and aged rats, 24 hours post-training in a fear conditioning paradigm (Monti et al., 2005). The differences between this study and the current set could be due to different molecular protocols, Monti and colleagues analysed the levels of BDNF using an enzyme-linked immunosorbent assay (ELISA).

Activation of the 5-HT_6 receptor has been shown to increase BDNF (de Foubert et al., 2007), one explanation for this is that the 5-HT_6 receptor is a GPCR and

activates adenylyl cyclase, this in turn activates the transcription factor of BDNF, CREB, hence increasing levels of the neurotrophin. An indirect mechanism involving glutamatergic neurotransmission may also account for the effect observed by de Foubert and colleagues. As BDNF is part of the signalling pathway of the NMDA receptor, therefore, an increase of glutamate, and hence excitatory transmission, may increase BDNF levels.

5-HT_6 receptor expression was detected in the current experiments using an antibody characterised in our group (Bentley., 1999; Woolley., 2002). The antibody was against a synthetic peptide sequence from the N-terminus of the rat 5-HT_6 receptor (anti-3-15YC serum). To determine specificity of the antibody to the 5-HT_6 receptor, a search of the BLAST database was performed, this failed to identify any homology between 3-15 (PEPGPVNSSTPAW) and other mammalian proteins, therefore the current studies results are due to selective binding of the 5-HT_6 receptor. The current set of studies found no difference between groups in 5-HT_6 receptor protein expression. Little literature is available on expression of 5-HT_6 receptor following fear associated learning and memory; one group utilised an autoshaping task and found receptor mRNA expression to be reduced following training (Huerta-Rivas et al., 2010). MK-801 has failed to have an impact on hippocampal 5-HT_6 receptor expression in previous studies (Healy and Meador-Woodruff, 1999; Huerta-Rivas et al., 2010), but did cause a reduction in striatal levels (Healy and Meador-Woodruff, 1999).

It is noteworthy that initial *in vitro* studies found rat 5-HT_6 receptors became desensitised with exposure to 5-HT in cell lines (Max et al 1995). The selective

5-HT_6 receptor agonist, WAY 181187, induced reproducible GABA release, measured by microdialysis, suggesting desensitisation does not occur *in vivo* (Schechter et al., 2008), and therefore the lack of changes observed in 5-HT_6 receptor levels cannot be due to this. Although changes have been observed in previous studies of BDNF and 5-HT_6 receptor expression, mRNA expression changes may not be a direct indication of protein changes.

It should be noted that immunoblots identified a strong band at 26 kDa for BDNF and 90 kDa for 5-HT_6 receptor (Figure 5-2). In regards to the 90 kDa band with the 5-HT_6 receptor it was not present if membrane was incubated with pre-immune serum (Figure 5-1, Woolley 2002). This suggests that the band seen at 90 kDa is not due to non-specific binding but is a homodimer of the 5-HT_6 receptor protein. The cDNA sequence of the rat 5-HT_6 receptor predicted a 46 kDa protein (Kohen et al 1996), due to the cysteine residues present in the receptor it is possible that the receptor exists as a dimer. The current set of studies prepared the tissue homogenates in solutions containing β-mercaptoethanol, this only removes disulphides and dimers are held together with hydrophobic forces, hence the strong bands at the molecular weight of the dimers observed in the current studies must be SDS stable.

5.5 Key Findings

No statistically significant differences in hippocampal levels of BDNF or 5-HT_6 receptor protein expression levels were observed 24 hours post CER training. This is most likely due to timing of tissue collection and future studies would

benefit from identifying the optimal time to collect tissue. Both SB-271046 and EMD 386088 elicited similar trends on BDNF expression, therefore the paradoxical behavioural effects could be mediated by a common effect on this protein. Future studies could analyse initial steps in the underlying molecular pathway for any differences. Little effect was observed on 5-HT_6 receptor protein expression following any treatment group, again the antagonist and agonist elicited similar effects upon expression levels. Future work into downstream effects of inhibition and activation of 5-HT_6 receptor are required to elucidate how drugs acting on this receptor work.

6 General Discussion

The main aim of this thesis was to perform a pharmacological characterisation and validation of a fear conditioning paradigm in rats. Secondly this paradigm was utilised to elucidate the role of the 5-HT_6 receptor in a fear conditioned learning and memory, by analysing the effects of the selective antagonist and agonists, initially alone and then to reverse cholinergic and glutamatergic deficits in the learning and memory processes. Finally BDNF and 5-HT_6 receptor protein levels within the hippocampus were analysed following CER to attempt to determine the downstream intracellular mechanisms involved in 5-HT_6 receptor signalling using the antagonists and agonists.

6.1 Summary of Findings and Discussion

6.1.1 Optimisation of CER

Experiments described in Chapter 2 were performed to optimise and validate a robust and reliable CER paradigm in rats. Previous studies found that if shocks were administered immediately following exposure to the novel context in fear conditioning paradigms there is little, or no, freezing behaviour (Fanselow, 1986), referred to as the immediate shock deficit. This is due to the subjects failing to form an association between the contextual CS and shock delivered during fear conditioning; hence, studies performed throughout current thesis allowed the rat to choose entry into the dark side of the box and following 30

seconds exploration of this side received the first shock. The initial study analysed the effects of one, three and five CS-US administrations on 24 hour memory retention. As expected when the number of CS-US associations was increased the freezing behaviour elicited during testing increased, three CS-US associations induced a significant freezing response, and although no statistical difference in freezing was observed between 3 and 5 CS-US associations there was an apparent increase in duration. Due to restrictions on the Home Office project licence (which current work was performed under) the effect of increased shock intensity (further than 0.4mA) could not be analysed. Another group analysed effects of 0.2, 0.4 and 1.0 mA, and found increasing shock intensity caused an increase in freezing behaviour (Cordero et al., 1998). This study supports the use of 0.4 mA shock intensity utilised throughout the current thesis, as it produced a robust reproducible freezing response during the test trial indicative of the expected association between context and cue.

Further validation work on drug-naive adult rats found the CER-induced memory underwent extinction when rats were re-introduced to the training chamber in the absence of further US administration. Hence rats were re-learning the environment was no longer aversive, and, therefore, were not eliciting as strong a freezing response when placed back in the training context. Extinction of memory can be utilised as a model to study fear inhibition in human, which is thought to be affected in post-traumatic stress disorder and panic attacks. The strength of the association between the context and shock is reduced each time the CS is presented without the US. Previous studies have analysed extinction and shown the original memory still remains intact but that subjects had learnt a

new association (i.e. CS = no US), this is supported by renewal, spontaneous recovery and reinstatement (Bouton et al., 2006; Ji and Maren, 2007). Examination of extinction processes was not a major aim of the work performed throughout this thesis, although it was interesting to note that extinction of CER was demonstrable in the current paradigm.

The majority of experiments in this thesis tested memory retention 24 hours post-training. Previous studies have analysed the effects of increased time between training and retention memory testing (Anagnostaras et al., 2001). The study in Chapter 2 tested retention of CER memory between 24-96 hours following training, the CER-induced freezing behaviour elicited 96 hours post-training, without further exposure to the context, was of the same magnitude as that elicited 24 hours post-training. Other studies, analysing neuroanatomical components of fear conditioning, found rats froze in response to re-exposure to training contexts up to 50 days following training (Anagnostaras et al., 1999a; Kim and Fanselow, 1992). Furthermore, lesion studies have illustrated the time-limited dependence of fear conditioning memories upon the hippocampus, such that lesions made 1 day following training causing significant memory deficits which are attenuated if lesions were made 7-28 days following training. This indicates that fear conditioning induces a strong memory, which over a certain time period becomes independent of the hippocampus. Consistent with this observation the data obtained with the paradigm used throughout this thesis showed that the freezing response induced at 96 hours post-training was of the same intensity as that observed in a 24 hour retention test.

The final validation/optimisation study in drug-naive adults rats analysed the effects of contextual CS and cue CS administration in a within subjects test. Rats froze to the contextual CS, as observed in the previous studies. Interestingly the same subjects elicited no CER in a novel context until the auditory and visual cue CS (identical to that administered on training day) was presented. Although the CER behaviour elicited by rats was identical irrespective of the modality of the CS administered during the test trial, other research groups have found that the underlying mechanisms involved in these behaviours are different (Phillips and Ledoux, 1992). Lesion studies have found that damage to the hippocampus induces contextual fear deficits but has no effect on the CER elicited following cue CS (Kim and Fanselow, 1992). Nonetheless, lesions to the amygdala cause deficits in both contextual and cue associated memories (Maren et al., 1996). These previous studies provided evidence that the hippocampus plays a temporary role in the acquisition/consolidation processes of contextual fear memories but does not affect cue-associated memories, whereas the amygdala is involved in both (Anagnostaras et al., 2001; Kim and Fanselow, 1992; Maren et al., 1996). Various studies have found different results to those stated above, with electrolytic lesions causing severe impairments (Kim et al., 1993) and pre-training neurotoxic lesions causing no deficits (Maren et al., 1997) in the response, this is most likely due to spread of the electrolytic lesions on surrounding axons. Also, neurotoxic lesions made following training cause deficits, whereas the same toxin given prior has no effect (Maren et al., 1997), supporting different forms of acquisition of contextual and cue memories. For instance, a healthy rat would utilise a unified representation of the context to form a CS-US, which would be prone to disruption with post-training lesions.

Whereas, if the hippocampus was not utilised during training, rats would associate simple elements that make up the unified contextual stimuli, with a shock (Maren, 2001). Generally it is believed that the hippocampus and amygdala play integral roles in acquiring and processing fear conditioning, with the amygdala forming and storing the CS-US associations, whether the unimodal cue-CS or multimodal contextual-CS is used (Maren, 2001). Subsequent studies in this thesis focussed on CER behaviours in response to contextual CS presentations and examined accompanying proteomic changes within the hippocampus given its known involvement in this response.

Once optimised, pharmacological compounds known to enhance or impair memory were administered to determine their effects on fear-motivated of learning and memory. As with many previous studies administration of the muscarinic acetylcholine receptor antagonist, scopolamine, impaired memory when administered prior to training; disrupting the acquisition of CER memory (Anagnostaras et al., 1999b; Foley et al., 2004). It is known that cholinergic neurotransmission is involved in cognitive processing (Everitt and Robbins, 1997; Pepeu and Giovannini, 2009), and that there is a decline in cholinergic neurotransmission associated with cognitive decline (Perry et al., 1992; Perry et al., 1978). Microdialysis studies have found that during memory acquisition of learning and memory tasks there is an increase in ACh levels within hippocampus (Nail-Boucherie et al., 2000; Orsetti et al., 1996). In a contextual fear conditioning paradigm, where rats are placed directly in a context and administered shocks (with no light or tone stimuli) and memory retention was tested 24 hours later, there was an increase in hippocampal ACh levels in both

conditioned (shock treated) and unconditioned (no shock controls), whereas during the retention trial only the conditioned rats elicited an increase in hippocampal ACh levels (Nail-Boucherie et al., 2000). In the present studies scopolamine was administered prior to training/acquisition, preventing ACh effects on receptors, therefore preventing the mnemonic aid of increased ACh on forming a strong CS-US association. Thus, 24 hours post-training there is a reduction in freezing behaviour indicative of a memory deficit. Following this the effects of known pro-cognitive drugs were analysed. Two AChEIs, donepezil and galantamine, had no effect upon CER-induced memory when given alone. In these studies the vehicle shock treated rats froze for a long duration, as there was no natural forgetting during the 24 hour retention trial the drugs were unable to further enhance memory in healthy adolescent rats. When tested on a scopolamine-induced deficit, donepezil reversed the attenuation of freezing, probably by increasing synaptic ACh levels, as shown by other groups (Lindner et al., 2006). These initial pharmacological studies provided strong evidence that the cholinergic system plays a vital role in the processing of CER memories.

These studies provided evidence that the CER paradigm optimised within our laboratory induced a strong freezing response, indicative of learning and memory, 24 hours post-training that could be manipulated pharmacologically to impair and enhance memory.

6.1.2 Effects of 5-HT$_6$ receptor on CER

Since its discovery, the 5-HT$_6$ receptor has generated a lot of interest, in particular as a number of antipsychotic and antidepressant drugs have affinity for

this receptor, and the unusual paradoxical pro-cognitive effect of both agonists and antagonists for this receptor in cognitive tasks (Chapter 1, Fone 2008; King et al 2008). The current thesis utilised the CER paradigm to determine the role of the 5-HT_6 receptor in fear motivated learning.

As mentioned in Chapter 1, much research has focussed on the effects of 5-HT_6 receptor ligands on pre-clinical cognitive behavioural tasks (summarised in Table 6-1). Of particular interest some reports using recently developed $5HT_6$ receptor agonists show that they have similar pro-cognitive effects to the antagonists. Ligands that act upon the 5-HT_6 receptor do not appear to have mnemonic affects in healthy adult subjects, other than facilitating the ED shift in an attentional set shifting task (Burnham et al., 2010). However 5-HT_6 receptor antagonists alleviate the deficits of natural forgetting and pharmacological deficits in a variety of learning and memory tasks (Fone 2008, Table 6-1). Little data is available regarding the effects of 5-HT_6 receptor compounds on CER, the current studies revealed interesting responses to the 5-HT_6 antagonists when given alone. If administered prior to training, SB-271046 attenuated freezing behaviour, this could be due to the anti-nociceptive properties associated with 5-HT_6 receptor antagonists (Finn et al., 2007). Hence, in the current case, rats pre-treated with SB-271046 may have had an altered perception of the shock and, therefore, made less association between the context-CS and US. This was supported by the fact that the SB-271046 treated rats did not vocalise during the training session as seen in the vehicle shock treated rats although this was a subjective view of the observer and not objectively quantified. In contrast, when administered immediately following training, or prior to the 24 hour retention trial, SB-271046

Paradigm	Form of learning and memory	5-HT$_6$ receptor antagonist	5-HT$_6$ receptor agonist
NOR	Recognition	Drug-induced memory deficits: • *Scopolamine:* Ro 04-6790 [1] Ro 4368554 [3, 4] SB-399885 [6] • *MK-801*: Ro 04-6790 [2, 5] • *TRP depletion:* Ro 4368554 [4] Natural forgetting: Ro 04-6790 [2, 9] SB-271046 [2, 9] Aged deficit: BGC20-761 [7] Ro 04-6790 [19]	Drug-induced memory deficits: • *Scopolamine:* E-6801 [9] Natural forgetting: E-6801 [9] EMD 386088 [9] R-13*c* [25]
Social Recognition	Social memory	Drug-induced memory deficits: • *WAY 181187:* SB-271046 [8] SB-258585 [8] • *Scopolamine:* SB-271046 [8] SB-258585 [8] BGC20-761 [7] Ro 4368554 [3] Natural forgetting: SB-271046 [8] SB-258585 [8]	WAY 181187 impaired social recognition[8]
Morris Water Maze	Spatial	Improved acquisition: SB-271046 [13] Improved retention: Ro 04-6790 [10] SB-271046 [17] SB-357134 [11, 17] Aged deficit SB-271046 [12] SB-399885 [6]	
Autoshaping task	Associative	Improved consolidation: Ro 04-6790 [22] SB-357134 [24] SB-399885 [23, 24] Drug-induced memory deficits: • *Scopolamine:* Ro 04-6790 [22] SB-399885 [24] • *MK-801:* SB-399885 [24]	

Attentional set shifting	Executive	Improved ED/ID shift: SB-271046 [14]	Improved ED shift: WAY 181187 [15]
Prepulse inhibition of startle	Sensory processing	Drug-induced memory deficits: • *Apomorphine:* Ro 4368554 [20] • *D-amphetamine:* SB-271046 [21]	
Passive Avoidance	Associative	Drug-induced memory deficits: • *Scopolamine:* Ro 04-6790 [16] Ro 4368554 [3] SB-271046 [12] 4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine [18]	

Table 6-1- Overview of the effects of 5-HT$_6$ receptor antagonists and agonists on either aged-, time-, or drug-induced memory deficits in various pre-clinical cognitive paradigms. [1] Woolley *et al*., 2003 [2] King *et al*., 2004 [3] Schreiber *et al*., 2007 [4] Lieben *et al*., 2005 [5] Pitsikas *et al*., 2008 [6] Hirst *et al*., 2006 [7] Mitchell *et al.,* 2006 [8] Loiseau *et al*., 2008 [9] Kendall *et al*., 2010 [10] Woolley *et al*., 2001 [11] Stean *et al.,* 2002 [12] Foley *et al*., 2004 [13] Marcos *et al*., 2008 [14] Hatcher *et al*., 2005 [15] Burnham *et al*., 2010 [16] Bos *et al*., 2001 [17] Rogers and Hagan., 2001 [18] Riemer *et al.,* 2003 [19] King., 2006 [20] Mitchell and Neumaier., 2008 [21] Pouzet *et al*., 2002 [22] Menenses *et al.,* 2001 [23] Meneses *et al*., 2007 [24] Perez-Garcia *et al.,* 2005 [25] King *et al.,* 2006 (abstract).

had little effect upon CER-induced freezing behaviour. The 5-HT_6 receptor agonists, EMD 386088 and E-6801, were only tested immediately following training, as this time point was selected following the antagonist studies, allowing a direct comparison between antagonist and agonist effects to be observed. When given alone neither of the agonists had any effect upon CER-induced freezing behaviour.

As there were no effects on drug-naive adult rats, pharmacological deficits in memory were induced allowing the effects of these 5-HT_6 receptor ligands on cholinergic and glutamatergic induced deficits to be analysed. In other paradigms both antagonists and agonists can reverse cholinergic and glutamatergic memory deficits (Kendall et al., 2010; King et al., 2004; Perez-Garcia and Meneses, 2005; Woolley et al., 2003). The literature provides evidence of a link between the 5-HT_6 receptor and central cholinergic neurotransmission. Initial studies found 5-HT_6 receptor directed antisense oligonucleotides (AO) directed against the initiation codon region of the 5-HT_6 mRNA (Bourson et al., 1995; Sleight et al., 1996) and the first antagonists, to induce a stretching and yawning behaviour that was abolished with muscarinic antagonists, atropine and scopolamine (Bentley et al., 1999; Bourson et al., 1995; Sleight et al., 1998), suggesting 5-HT_6 receptor antagonist may enhance ACh levels. Further research found 5-HT_6 receptor antagonists inhibited atropine- and scopolamine-induced ipsilateral rotations (Bourson et al., 1998). Recent neurochemical data support the link between the 5-HT_6 receptor and central cholinergic neurotransmission, with increases in ACh levels within brain areas associated with memory following treatment with antagonists (Riemer et al.,

2003; Shirazi-Southall et al., 2002). In the current set of studies the antagonist, SB-271046, and agonist, E-6801, reversed scopolamine-induced deficit in CER, with EMD 386088 just failing to reach significance but showing a clear trend towards reversal. These results suggest that administration of either SB-271046, EMD 386088 or E-6801, immediately following CER training increases ACh levels to reverse the blockade induced from scopolamine. This proposal fits for the antagonists as microdiaylsis studies have shown an increase in ACh levels (Riemer et al., 2003; Shirazi-Southall et al., 2002), although the underlying mechanisms are unknown. The possible reasons for the paradoxical effects of antagonists and agonists at the 5-HT_6 receptor are discussed below.

There is evidence supporting a link between the 5-HT_6 receptor and glutamatergic neurotransmission. Memory deficits, induced via administration of the NMDA receptor antagonist MK-801, in paradigms such as NOR and autoshaping were reversed following treatment with 5-HT_6 receptor antagonists (King et al., 2004; Perez-Garcia and Meneses, 2005). These pre-clinical behavioural paradigms provided initial evidence of a link between the 5-HT_6 receptor and the glutamate system which was supported with neurochemical findings of increased glutamate levels following administration of 5-HT_6 receptor antagonists (Dawson et al., 2000, 2001; Woolley 2002). Studies performed in Chapters 3 and 4 found CER freezing to be reduced by pre-training administration of MK-801, indicating a role for glutamate in this form of learning and memory. This glutamatergic-induced memory deficit was reversed following acute systemic treatment with either SB-271046, EMD 386088 or E-6801. As previously mentioned MK-801 has induced memory deficits in various pre-

clinical paradigms (Csernansky et al., 2005; Nilsson et al., 2007) but this is the first study to show that both 5-HT_6 receptor antagonists and agonists can reverse a glutamatergic deficit in CER.

6.2 Paradoxical effects

Following the development of selective 5-HT_6 receptor antagonists (Section 1.3.5.6) it was found that inhibition of this receptor had pro-cognitive effects in various pre-clinical paradigms (Section 1.3.5.16, Table 6-1). Therefore, initial hypotheses regarding the effects of 5-HT_6 receptor agonists were that activation of the receptor would have a negative effect upon learning and memory. Microdiaylsis studies found that treatment with the selective 5-HT_6 receptor agonists, WAY 181187 and WAY 208466, increased GABA levels in the dorsal hippocampus, striatum, frontal cortex and amygdala, which was blocked with the antagonist SB-271046 (Schechter et al., 2008). Glutamate release was attenuated in hippocampal slices following treatment with 5-HT_6 receptor agonists, although this was not shown *in vivo* (Schechter et al., 2008). These data supported the hypothesis of opposing effects exerted by the agonist and antagonist on learning and memory. Initial studies supported this hypothesis, with agonists causing an impairment in social recognition (Loiseau et al., 2008) and short- and long-term memory in an autoshaping task (Meneses et al., 2008). However, recent data have shown that the 5-HT_6 receptor agonists elicit pro-cognitive effects in the attentional set-shifting task (Burnham et al., 2010) and NOR (Kendall et al., 2010). Interestingly our group found that the agonist, E-6801, and antagonist, SB-271046, acted synergistically in the NOR paradigm (Kendall et al., 2010).

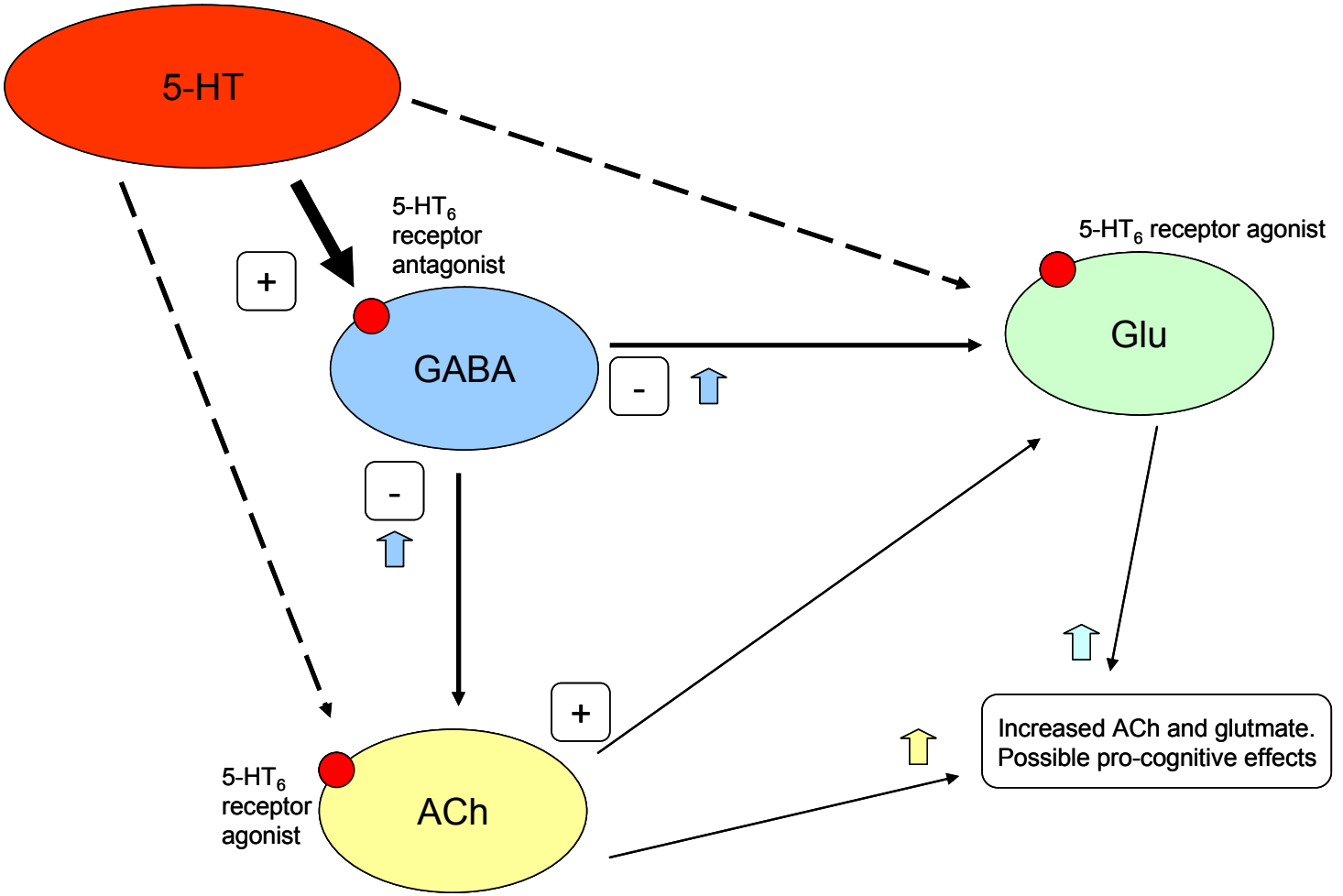
Similar paradoxical findings in response to antagonists and agonists have been observed in the tail suspension and forced swim test tasks (Svenningsson et al., 2007; Wesolowska and Nikiforuk, 2007). Studies on the treatment of obesity and feeding behaviour have also displayed paradoxical effects, with both 5-HT_6 receptor antagonists and agonists significantly reducing food intake and body weight (Fisas et al., 2006; Heal et al., 2008; Woolley et al., 2001).

With these results and those from the current thesis, it appears that antagonists and agonists acting at the 5-HT_6 receptor can induce similar behavioural responses, although the underlying mechanisms involved in these paradoxical behavioural effects are unknown to date. There are some possibilities as to how these effects occur, antagonists and agonists may act on separate underlying mechanisms. *In vitro* studies have found that 5-HT_6 receptors expressed in cell lines are positively coupled to cAMP production (Kohen et al., 1996; Monsma et al., 1993; Ruat et al., 1993). A recent study *in vivo* identified an interaction between the human 5-HT_6 receptor and Fyn-tyrosine kinase (Yun et al., 2007). The agonists and antagonists therefore could act differentially upon these two separate underlying pathways. Further work into the effects of 5-HT_6 receptor ligands on these pathways is required. In Chapter 5 experiments were performed to elucidate any effects upon BDNF or the 5-HT_6 receptor itself, which may have contributed to the paradoxical effects observed. Unfortunately the results failed to show any significant differences between groups, but some interesting trends in the data were observed. CER training caused an increase in BDNF expression, which was reduced with both scopolamine and MK-801 treatment, both the antagonist, SB-271046, and agonist, EMD 386088, increased BDNF expression

following the cholinergic and glutamatergic induced deficits. 5-HT_6 receptor expression was decreased with CER training, this was further reduced with scopolamine, SB-271046 and EMD 386088 increased the expression following scopolamine. MK-801 caused an increase in 5-HT_6 receptor expression, whilst SB-271046 further enhanced this expression, EMD 386088 reduced the relative intensity observed. Significant differences may not have been observed in the studies due to the tissue being taken 24 hours after CER training, other groups have shown differences in BDNF expression after 0.5 to 2 hours post-training returning to basal levels at 4-6 hours (Barrientos et al., 2004; Hall et al., 2000), therefore any changes that may have occurred in the current studies may not have been observed at 24 hours post-training. Future work on testing the proteomic changes at various time points following CER training would be of great interest, and would help elucidate the underlying mechanisms involved in this learning and memory process.

Another explanation for the paradoxical behavioural effects observed following treatment with antagonists and agonists could be due to the location of 5-HT_6 receptors. It is known from dual labelled immunohistochemistry that there is little co-existence between 5-HT_6 receptors and ChAT, and lesions of the cholinergic system via administration of the immunotoxin 192-IgG-Saporin had no effect on 5-HT_6 receptor mRNA or protein levels (Marcos et al., 2006; Woolley et al., 2004). Further work found extensive co-existence of the receptor with GAD-67, providing evidence of localisation on GABAergic neurones (Woolley et al., 2004).

Figure 6-1- Schematic of proposed mechanism of paradoxical effects induced via 5-HT_6 receptor antagonists and agonists within the hippocampus. Serotonergic inputs (red) directly onto cholinergic (yellow) or glutamatergic (green) or indirectly onto GABAergic interneurones (blue). Dashed arrow = little serotonergic input, block black arrow = high serotonergic input, block colour arrows = increase in relative neurotransmitter.


5-HT
5-HT6 receptor antagonist
+
GABA
-
5-HT6 receptor agonist
Glu
-
+
5-HT6 receptor agonist
ACh
Increased ACh and glutmate.
Possible pro-cognitive effects

Therefore, within the hippocampus, agonists may act upon the few 5-HT_6 receptors that are located directly on the glutamatergic and/or cholinergic neurones, which under normal condition receive little serotonergic input, whereas the antagonists act on 5-HT_6 receptors located on upstream inhibitory GABAergic neurones, that receive active serotonergic input, which disinhibit glutamate and ACh release (Figure 6-1). Therefore both agonist and antagonist treatment would induce an increase in glutamate and ACh levels, accounting for their pro-cognitive effects as observed in the current studies.

6.3 Future Work

These studies have provided evidence that 5-HT_6 receptors play a role in CER, with literature showing their pro-cognitive effects on other forms of learning and memory, therefore it is of great interest to understand the mechanisms of the drugs that act upon it. As the antagonists and agonists at the 5-HT_6 receptor elicited paradoxical effects on CER, and other pre-clinical cognitive paradigms, it is clear that future investigations are required to elucidate the underlying mechanisms following activation and/or inhibition of the receptor.

As the development of selective 5-HT_6 receptors is very recent further behavioural and neurochemical work is required to determine the effects of these drugs upon various models of learning and memory. For instance initial studies found the agonists to impair memory in social recognition and an autoshaping task, whereas recent literature provides evidence of a pro-cognitive effect, further

work with more selective agonists will give a clearer understanding to the behavioural effects of activating 5-HT_6 receptors.

Following the findings in the current thesis, microdialysis studies on the effects of ACh and glutamate release following acute treatment with 5-HT_6 receptor ligands would be highly beneficial in determining the paradoxical mnemonic effects observed in learning and memory paradigms. Also literature has found that there is an interaction between 5-HT_6 receptors and fyn tyrosine kinase, and it is known that 5-HT_6 receptors are positively coupled to adenylyl cyclase. Therefore, future proteomic analysis on the effects of either antagonists and agonists on various pathways, and expression of certain proteins, such as fyn tyrosine, would greatly aid the understanding of underlying events following activation/inhibition of the 5-HT_6 receptor. This could be achieved using Western blot, or 2-dimensional gel electrophoresis, which separates proteins by their isoelectric point and molecular weight. Utilising this technique may also find novel proteins altered by 5-HT_6 receptor ligands.

Due to the pro-cognitive effects elicited in CER and other pre-clinical paradigms following treatment with 5-HT_6 receptor ligands much interest surrounds these compounds on their therapeutic uses in treating cognitive dysfunction in AD, schizophrenia, and obesity. Current antipsychotic treatments can induce weight gain in patients; therefore, pre-clinical findings of reduced body weight and food intake with both antagonists and agonists (Fisas et al., 2006; Woolley et al., 2004) are of great interest for clinical studies, with compounds entering phase I clinical trials for the treatment of obesity. Due to the detrimental effects of AD and

schizophrenia on multiple neurotransmitter systems it is believed that pharmacological therapies that modulate several neurotransmitters would be more beneficial than a single target. Hence, the pre-clinical findings that both antagonists and agonists acting on the 5-HT_6 receptor modulate multiple systems is of great interest, especially alongside the behavioural findings of reduced body weight, anti-depressant actions, and pro-cognitive effects. Several clinical trials are now being performed testing the effects of 5-HT_6 receptor ligands on obesity, AD and schizophrenia (Table 1-2, Heal et al 2008; Upton et al 2010), with initial reports illustrating similar beneficial effects to those observed in pre-clinical studies. Following this it would be of great interest to observe the synergistic effects of 5-HT_6 receptor ligands with current drug treatments for schizophrenia, AD and obesity, as initial pre-clinical studies have found a beneficial effect of combination treatments.

7 References

Abraham, W. C., Williams, J. M. (2008). LTP maintenance and its protein synthesis-dependence. *Neurobiology of Learning and Memory* **89**, 260-268.

Ahi, J., Radulovic, J., Spiess, J. (2004). The role of hippocampal signaling cascades in consolidation of fear memory. *Behavioural Brain Research* **149**, 17-31.

Aigner, T. G. (1995). Pharmacology of memory: Cholinergic-Glutamatergic interactions. *Current Opinion in Neurobiology* **5**, 155-160.

Alcalde, E., Mesquida, N., Lopez-Perez, S., Frigola, J., Merce, R. (2009). Indene-Based Scaffolds. 2. An Indole-Indene Switch: Discovery of Novel Indenylsulfonamides as 5-HT_6 Serotonin Receptor Agonists. *Journal of Medicinal Chemistry* **52**, 675-687.

Alvarez-Alvarez, M., Galdos, L., Fernandez-Martinez, M., Gomez-Busto, F., Garcia-Centeno, V., Arias-Arias, C., Sanchez-Salazar, C., Rodriguez-Martinez, A. B., Zarranz, J. J., de Pancorbo, M. M. (2003). 5-Hydroxytryptamine 6 receptor (5-HT_6) receptor and apolipoprotein E (ApoE) polymorphisms in patients with Alzheimer's disease in the Basque Country. *Neuroscience Letters* **339**, 85-87.

Anagnostaras, S. G., Gale, G. D., Fanselow, M. S. (2001). Hippocampus and contextual fear conditioning: recent controversies and advances. *Hippocampus* **11**, 8-17.

Anagnostaras, S. G., Maren, S., Fanselow, M. S. (1999a). Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: Within-subjects examination. *Journal of Neuroscience* **19**, 1106-1114.

Anagnostaras, S. G., Maren, S., Sage, J. R., Goodrich, S., Fanselow, M. S. (1999b). Scopolamine and Pavlovian fear conditioning in rats: dose-effect analysis. *Neuropsychopharmacology* **21**, 731-744.

Azmitia, E. C., Segal, M. (1978). Autoradiographic analysis of differential ascending projections of dorsal and median raphe nuclei in rat. *Journal of Comparative Neurology* **179**, 641-667.

Barnes, C. A., Meltzer, J., Houston, F., Orr, G., McGann, K., Wenk, G. L. (2000). Chronic treatment of old rats with donepezil or galantamine: Effects on memory, hippocampal plasticity and nicotinic receptors. *Neuroscience* **99**, 17-23.

Barnes, N. M., Sharp, T. (1999). A review of central 5-HT receptors and their function. *Neuropharmacology* **38**, 1083-1152.

Barondes, S. H., Cohen, H. D. (1968). Arousal and the conversion of "short-term" to "long-term" memory. *Proceedings of the National Academy of Sciences U.S.A* **61**, 923-929.

Barrientos, R. M., Sprunger, D. B., Campeau, S., Watkins, L. R., Rudy, J. W., Maier, S. F. (2004). BDNF mRNA expression in rat hippocampus following contextual learning is blocked by intrahippocampal IL-1beta administration. *Journal of Neuroimmunology* **155**, 119-126.

Bartus, R. T., Dean, R. L., Beer, B., Lippa, A. S. (1982). The cholinergic hypothesis of geriatric memory dysfunction. *Science* **217**, 408-417.

Bear, M. F. (1997). How do memories leave their mark? *Nature* **385**, 481-482.

Bear, M. F., Abraham, W. C. (1996). Long-term depression in hippocampus. *Annual Review of Neuroscience* **19**, 437-462.

Bentley, J. C. (1999). 5-HT_6 receptor function in the CNS. *Thesis, University of Nottingham*.

Bentley, J. C., Bourson, A., Boess, F. G., Fone, K. C. F., Marsden, C. A., Petit, N., Sleight, A. J. (1999). Investigation of stretching behaviour induced by the selective 5-HT_6 receptor antagonist, Ro 04-6790, in rats. *British Journal of Pharmacology* **126**, 1537-1542.

Blakely, R. D., Berson, H. E., Fremeau, R. T., Jr., Caron, M. G., Peek, M. M., Prince, H. K., Bradley, C. C. (1991). Cloning and expression of a functional serotonin transporter from rat brain. *Nature* **354**, 66-70.

Blanchard, D. C., Blanchard, R. J. (1972). Innate and conditioned reactions to threat in rats with amygdaloid lesions. *Journal of Comparative and Physiological Psychology* **81**, 281-290.

Blanchard, R. J., Blanchard, D. C. (1969). Passive and active reactions to fear eliciting stimuli. *Journal of Comparative and Physiological Psychology* **68**, 129-135.

Bliss, T. V., Collingridge, G. L., Laroche, S. (2006). Neuroscience. ZAP and ZIP, a story to forget. *Science* **313**, 1058-1059.

Bliss, T. V. P., Collingridge, G. L. (1993). A synaptic model of memory-long-term potentiation in the hippocampus. *Nature* **361**, 31-39.

Bliss, T. V. P., Lomo, T. (1973). Long lasting potentiation of synaptic transmission in dentate area of anesthetized rabbit following stimulation of perforant path. *Journal of Physiology-London* **232**, 331-356.

Boast, C., Bartolomeo, A. C., Morris, H., Moyer, J. A. (1999). 5HT antagonists attenuate MK801-impaired radial arm maze performance in rats. *Neurobiology of Learning and Memory* **71**, 259-271.

Boess, F. G., Monsma, F. J., Meyer, V., Zwingelstein, C., Sleight, A. J. (1997). Interaction of tryptamine and ergoline compounds with threonine 196 in the ligand binding site of the 5-hydroxytryptamine(6) receptor. *Molecular Pharmacology* **52**, 515-523.

Boess, F. G., Monsma, F. J., Sleight, A. J. (1998a). Identification of residues in transmembrane regions III and VI that contribute to the ligand binding site of the serotonin 5-HT_6 receptor. *Journal of Neurochemistry* **71**, 2169-2177.

Boess, F. G., Riemer, C., Bos, M., Bentley, J., Bourson, A., Sleight, A. J. (1998b). The 5-hydroxytryptamine6 receptor-selective radioligand [3H]Ro 63-0563 labels 5-hydroxytryptamine receptor binding sites in rat and porcine striatum. *Molecular Pharmacology* **54**, 577-583.

Bolles, R. C. (1970). Species-specific defense reaction and avoidance learning. *Psychological Review* **77**, 32-48.

Bontempi, B., Laurent-Demir, C., Destrade, C., Jaffard, R. (1999). Time-dependent reorganization of brain circuitry underlying long-term memory storage. *Nature* **400**, 671-675.

Bos, M., Sleight, A. J., Godel, T., Martin, J. R., Riemer, C., Stadler, H. (2001). 5-HT_6 receptor antagonists: lead optimisation and biological evaluation of N-aryl and N-heteroaryl 4-amino-benzene sulfonamides. *European Journal of Medicinal Chemistry* **36**, 165-178.

Bourson, A., Boess, F. G., Bos, M., Sleight, A. J. (1998). Involvement of 5-HT_6 receptors in nigro-striatal function in rodents. *British Journal of Pharmacology* **125**, 1562-1566.

Bourson, A., Borroni, E., Austin, R. H., Monsma, F. J., Jr., Sleight, A. J. (1995). Determination of the role of the 5-ht_6 receptor in the rat brain: a study using antisense oligonucleotides. *Journal of Pharmacology and Experimental Therapeutics* **274**, 173-180.

Bouton, M. E., Westbrook, R. F., Corcoran, K. A., Maren, S. (2006). Contextual and temporal modulation of extinction: behavioral and biological mechanisms. *Biological Psychiatry* **60**, 352-360.

Bowker, R. M., Westlund, K. N., Sullivan, M. C., Coulter, J. D. (1982). Organization of descending serotonergic projections to the spinal cord. *Progress in Brain Research* **57**, 239-265.

Bradley, P. B., Engel, G., Feniuk, W., Fozard, J. R., Humphrey, P. P. A., Middlemiss, D. N., Mylecharane, E. J., Richardson, B. P., Saxena, P. R. (1986). Proposals for the classification and nomenclature of functional receptors for 5-hydroxytryptamine. *Neuropharmacology* **25**, 563-576.

Brioni, J. D., Nagahara, A. H., McGaugh, J. L. (1989). Involvement of the amygdala GABAergic system in the modulation of memory storage. *Brain Research* **487**, 105-112.

Broadbent, N. J., Squire, L. R., Clark, R. E. (2004). Spatial memory, recognition memory, and the hippocampus. *Proceedings of the National Academy of Sciences U.S.A* **101**, 14515-14520.

Brodie, B. B., Pletscher, A., Shore, P. A. (1955). Evidence that serotonin has a role in brain function. *Science* **122**, 968.

Bromidge, S. M., Brown, A. M., Clarke, S. E., Dodgson, K., Gager, T., Grassam, H. L., Jeffrey, P. M., Joiner, G. F., King, F. D., Middlemiss, D. N., Moss, S. F., Newman, H., Riley, G., Routledge, C., Wyman, P. (1999). 5-Chloro-N-(4-methoxy-3-piperazin-1-yl- phenyl)-3-methyl-2-benzothiophenesulfon- amide (SB-271046): a potent, selective, and orally bioavailable 5-HT_6 receptor antagonist. *Journal of Medicinal Chemistry* **42**, 202-205.

Bromidge, S. M., Clarke, S. E., Gager, T. Griffith, K., Jeffrey, P. Jennings, A. J., Joiner, G. F., King, F. D., Lovell, P. J., Moss, S. F., Newman, H., Riley, G., Rogers, D., Routledge, C., Serafinowska, H., Smith, D. R. (2001). Phenyl Benzenesulfonamides are novel and selective 5-HT_6 antagonists: Identification of N-(2,5-Dibromo-3-fluorophenyl)-4-methoxy-3-piperazin-1-ylbenzenesulfonamide (SB-357134). *Bioorganic and Medicinal Chemistry Letters* **11**, 2843-2846.

Burnham, K. E., Baxter, M. G., Bainton, J. R., Southam, E., Dawson, L. A., Bannerman, D. M., Sharp, T. (2010). Activation of 5-HT_6 receptors facilitates attentional set shifting. *Psychopharmacology* **208**, 13-21.

Castren, E., da Penha Berzaghi, M., Lindholm, D., Thoenen, H. (1993). Differential effects of MK-801 on brain-derived neurotrophic factor mRNA levels in different regions of the rat brain. *Experimental Neurology* **122**, 244-252.

Cirulli, F., Berry, A., Chiarotti, F., Alleva, E. (2004). Intrahippocampal administration of BDNF in adult rats affects short-term behavioral plasticity in the Morris water maze and performance in the elevated plus-maze. *Hippocampus* **14**, 802-807.

Clark, C. T., Weissbach, H., Udenfriend, S. (1954). 5-Hydroxytryptophan decarboxylase-preparation and properties. *Journal of Biological Chemistry* **210**, 139-148.

Cole, D. C., Ellingboe, J. W., Lennox, W. J., Mazandarani, H., Smith, D. L., Stock, J. R., Zhang, G. M., Zhou, P., Schechter, L. E. (2005a). N-1-arylsulfonyl-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indole derivatives are potent and selective 5-HT_6 receptor antagonists. *Bioorganic & Medicinal Chemistry Letters* **15**, 379-383.

Cole, D. C., Lennox, W. J., Lombardi, S., Ellingboe, J. W., Bernotas, R. C., Tawa, G. J., Mazandarani, H., Smith, D. L., Zhang, G. M., Coupet, J., Schechter, L. E. (2005b). Discovery of 5-arylsulfonamido-3(pyrrolidin-2-ylmethyl)-1H-indole derivatives as potent, selective 5-HT_6 receptor agonists and antagonists. *Journal of Medicinal Chemistry* **48**, 353-356.

Cole, D. C., Stock, J. R., Lennox, W. J., Bernotas, R. C., Ellingboe, J. W., Boikess, S., Coupet, J., Smith, D. L., Leung, L., Zhang, G. M., Feng, X. D., Kelly, M. F., Galante, R., Huang, P. Z., Dawson, L. A., Marquis, K., Rosenzweig-Lipson, S., Beyer, C. E., Schechter, L. E. (2007). Discovery of N-1-(6-chloroimidazo[2,1-b][1,3]-thiazole-5-sulfonyl)tryptamine as a potent, selective, and orally active 5-HT_6 receptor agonist. *Journal of Medicinal Chemistry* **50**, 5535-5538.

Cooper, J.R., Bloom, F.E., Roth, R.H. (2003). The biochemical basis of neuropharmacology. 8th Edition: Oxford University Press; Oxford, U.K.

Cordero, M. I., Merino, J. J., Sandi, C. (1998). Correlational relationship between shock intensity and corticosterone secretion on the establishment and subsequent expression of contextual fear conditioning. *Behavioral Neuroscience* **112**, 885-891.

Courtney, C., Farrell, D., Gray, R., Hills, R., Lynch, L., Sellwood, E., Edwards, S., Hardyman, W., Raftery, J., Crome, P., Lendon, C., Shaw, H., Bentham, P. (2004). Long-term donepezil treatment in 565 patients with Alzheimer's disease (AD2000): randomised double-blind trial. *Lancet* **363**, 2105-2115.

Coyle, J. T., Price, D. L., Delong, M. R. (1983). Alzheimer's disease-A disorder of cortical cholinergic innervation. *Science* **219**, 1184-1190.

Csernansky, J. G., Martin, M., Shah, R., Bertchume, A., Colvin, J., Dong, H. X. (2005). Cholinesterase inhibitors ameliorate behavioral deficits induced by MK-801 in mice. *Neuropsychopharmacology* **30**, 2135-2143.

Dalley, J. W., Cardinal, R. N., Robbins, T. W. (2004). Prefrontal executive and cognitive functions in rodents: neural and neurochemical substrates. *Neuroscience and Biobehavioral Reviews* **28**, 771-784.

Davis, H. P., Squire, L. R. (1984). Protein-synthesis and memory- A review. *Psychological Bulletin* **96**, 518-559.

Dawson, L. A., Nguyen, H. Q., Li, P. (2000). In vivo effects of the 5-HT_6 antagonist SE-271046 on striatal and frontal cortex extracellular concentrations of noradrenaline, dopamine, 5-HT, glutamate and aspartate. *British Journal of Pharmacology* **130**, 23-26.

Dawson, L. A., Nguyen, H. Q., Li, P. (2001). The 5-HT_6 receptor antagonist SB-271046 selectively enhances excitatory neurotransmission in the rat frontal cortex and hippocampus. *Neuropsychopharmacology* **25**, 662-668.

Dawson, L. A., Nguyen, H. Q., Li, P. (2003). Potentiation of amphetamine-induced changes in dopamine and 5-HT by a 5-HT_6 receptor antagonist. *Brain Research Bulletin* **59**, 513-521.

de Foubert, G., O'Neill, M. J., Zetterstrom, T. S. (2007). Acute onset by 5-HT_6-receptor activation on rat brain brain-derived neurotrophic factor and activity-regulated cytoskeletal-associated protein mRNA expression. *Neuroscience* **147**, 778-785.

de Kloet, E. R., Joels, M., Holsboer, F. (2005). Stress and the brain: From adaptation to disease. *Nature Reviews Neuroscience* **6**, 463-475.

Dimitrova, D. S., Getova-Spassova, D. P. (2006). Effects of galantamine and donepezil on active and passive avoidance tests in rats with induced hypoxia. *Journal of Pharmacological Sciences* **101**, 199-204.

Dooley, M., Lamb, H. M. (2000). Donepezil - A review of its use in Alzheimer's disease. *Drugs & Aging* **16**, 199-226.

Duncan, R. J. S., Sourkes, T. L. (1974). Some enzymic aspects of production of oxidized or reduced metabolites of catecholamines and 5-hydroxytryptamine by brain tissues. *Journal of Neurochemistry* **22**, 663-669.

East, S. Z., Burnet, P. W. J., Kerwin, R. W., Harrison, P. J. (2002a). An RT-PCR study of 5-HT_6 and 5-HT_7 receptor mRNAs in the hippocampal formation and prefrontal cortex in schizophrenia. *Schizophrenia Research* **57**, 15-26.

East, S. Z., Burnet, P. W. J., Leslie, R. A., Roberts, J. C., Harrison, P. J. (2002b). 5-HT_6 receptor binding sites in schizophrenia and following antipsychotic drug administration: Autoradiographic studies with [I-125]SB-258585. *Synapse* **45**, 191-199.

Ehrlich, I., Humeau, Y., Grenier, F., Ciocchi, S., Herry, C., Luthi, A. (2009). Amygdala inhibitory circuits and the control of fear memory. *Neuron* **62**, 757-771.

Elks, M. L., Youngblood, W. W., Kizer, J. S. (1979). Synthesis and release of serotonin by brain slices: Effect of ionic manipulations and cationic ionophores. *Brain Research* **172**, 461-469.

Erspamer, V. (1954). Pharmacology of indolealkylamines. *Pharmacological Reviews* **6**, 425-487.

Erspamer, V., Asero, B. (1952). Identification of enteramine, the specific hormone of the enterochromaffic cell system, as 5-hydroxytryptamine. *Nature* **169**, 800-801.

Everitt, B. J., Robbins, T. W. (1997). Central cholinergic systems and cognition. *Annual Review of Psychology* **48**, 649-684.

Falck, B., Thieme, G., Hillarp, N. A., Torp, A. (1962). Fluorescence of catecholamines and related compounds condensed with formaldehyde. *Journal of Histochemistry & Cytochemistry* **10**, 348-354.

Fanselow, M. S. (1986). Associative vs topographical accounts of the immediate shock freezing deficit in rats-implications for the response selection rules governing species-specific defensive reactions. *Learning and Motivation* **17**, 16-39.

Fendt, M., Fanselow, M. S. (1999). The neuroanatomical and neurochemical basis of conditioned fear. *Neuroscience and Biobehavioral Reviews* **23**, 743-760.

Finn, D. P., Fone, K. C. F., Beckett, S. R. G., Baxter, J. A., Ansell, L., Marsden, C. A., Chapman, V. (2007). The effects of pharmacological blockade of the 5-HT_6 receptor on formalin-evoked nociceptive behaviour, locomotor activity and hypothalamo-pituitary-adrenal axis activity in rats. European *Journal of Pharmacology* **569**, 59-63.

Fisas, A., Codony, X., Romero, G., Dordal, A., Giraldo, J., Merce, R., Holenz, J., Heal, D., Buschmann, H., Pauwels, P. J. (2006). Chronic 5-HT_6 receptor modulation by E-6837 induces hypophagia and sustained weight loss in diet-induced obese rats. *British Journal of Pharmacology* **148**, 973-983.

Foley, A. G., Murphy, K. J., Hirst, W. D., Gallagher, H. C., Hagan, J. J., Upton, N., Walsh, F. S., Regan, C. M. (2004). The 5-HT_6 receptor antagonist SB-271046 reverses scopolamine-disrupted consolidation of a passive avoidance task and ameliorates spatial task deficits in aged rats. *Neuropsychopharmacology* **29**, 93-100.

Fone, K. C. (2008). An update on the role of the 5-hydroxytryptamine(6) receptor in cognitive function. *Neuropharmacology* **55**, 1015-1022.

Frankland, P. W., Bontempi, B., Talton, L. E., Kaczmarek, L., Silva, A. J. (2004a). The involvement of the anterior cingulate cortex in remote contextual fear memory. *Science* **304**, 881-883.

Frankland, P. W., Josselyn, S. A., Anagnostaras, S. G., Kogan, J. H., Takahashi, E., Silva, A. J. (2004b). Consolidation of CS and US representations in associative fear conditioning. *Hippocampus* **14**, 557-569.

Frassetto, A., Zhang, J., Lao, J. Z., White, A., Metzger, J. M., Fong, T. M., Chen, R. Z. (2008). Reduced sensitivity to diet-induced obesity in mice carrying a mutant 5-HT_6 receptor. *Brain Research* **1236**, 140-144.

Frederick, J. A., Meador-Woodruff, J. H. (1999). Effects of clozapine and haloperidol on 5-HT_6 receptor mRNA levels in rat brain. *Schizophrenia Research* **38**, 7-12.

Fuxe, K. (1965). Evidence for existence of monoamine neurons in central nervous system. 3. Monoamine nerve terminal. *Zeitschrift Fur Zellforschung Und Mikroskopische Anatomie* **65**, 573-596.

Gaddum, J. H., Picarelli, Z. P. (1957). 2 Kinds of tryptamine receptor. *British Journal of Pharmacology and Chemotherapy* **12**, 323-328.

Gale, G. D., Anagnostaras, S. G., Fanselow, M. S. (2001). Cholinergic modulation of pavlovian fear conditioning: effects of intrahippocampal scopolamine infusion. *Hippocampus* **11**, 371-376.

Gale, G. D., Anagnostaras, S. G., Godsil, B. P., Mitchell, S., Nozawa, T., Sage, J. R., Wiltgen, B., Fanselow, M. S. (2004). Role of the basolateral amygdala in the storage of fear memories across the adult lifetime of rats. *Journal of Neuroscience* **24**, 3810-3815.

Garcia-Alloza, M., Gil-Bea, F. J., Diez-Ariza, M., Chen, C., Francis, P. T., Lasheras, B., Ramirez, M. J. (2005). Cholinergic-serotonergic imbalance contributes to cognitive and behavioral symptoms in Alzheimer's disease. *Neuropsychologia* **43**, 442-449.

Garcia-Alloza, M., Hirst, W. D., Chen, C. P., Lasheras, B., Francis, P. T., Ramirez, M. J. (2004). Differential involvement of 5-$HT_{1B/1D}$ and 5-HT_6 receptors in cognitive and non-cognitive symptoms in Alzheimer's disease. *Neuropsychopharmacology* **29**, 410-416.

Gerard, C., El Mestikawy, S., Lebrand, C., Adrien, J., Ruat, M., Traiffort, E., Hamon, M., Martres, M. P. (1996). Quantitative RT-PCR distribution of serotonin 5-HT_6 receptor mRNA in the central nervous system of control or 5,7-dihydroxytryptamine-treated rats. *Synapse* **23**, 164-173.

Gerard, C., Martres, M. P., Lefèvre, K., Miquel, M. C., Vergé, D., Lanfumey, L., Doucet, E., Hamon, M., El Mestikawy, S. (1997). Immuno-localization of serontonin 5-HT_6 receptor-like material in the rat central nervous system. *Brain Research* **746**, 207-219.

Glennon, R. A., Lee, M., Rangisetty, J. B., Dukat, M., Roth, B. L., Savage, J. E., McBride, A., Rauser, L., Hufeisen, S., Lee, D. K. H. (2000). 2-Substituted tryptamines: Agents with selectivity for 5-HT_6 serotonin receptors. *Journal of Medicinal Chemistry* **43**, 1011-1018.

Gooney, M., Messaoudi, E., Maher, F. O., Bramham, C. R., Lynch, M. A. (2004). BDNF-induced LTP in dentate gyrus is impaired with age: analysis of changes in cell signaling events. *Neurobiology of Aging* **25**, 1323-1331.

Greenamyre, J. T., Young, A. B. (1989). Excitatory Amino Acids and Alzheimer's Disease. *Neurobiology of Aging* **10**, 593-602.

Hall, J., Thomas, K. L., Everitt, B. J. (2000). Rapid and selective induction of BDNF expression in the hippocampus during contextual learning. *Nature Neuroscience* **3**, 533-535.

Hamon, M., Doucet, E., Lefevre, K., Miquel, M. C., Lanfumey, L., Insausti, R., Frechilla, D., Del Rio, J., Verge, D. (1999). Antibodies and antisense oligonucleotide for probing the distribution and putative functions of central 5-HT_6 receptors. *Neuropsychopharmacology* **21**, S68-S76.

Hatcher, P. D., Brown, V. J., Tait, D. S., Bate, S., Overend, P., Hagan, J. J., Jones, D. N. C. (2005). 5-HT_6 receptor antagonists improve performance in an attentional set shifting task in rats. *Psychopharmacology* **181**, 253-259.

Heal, D. J., Smith, S. L., Fisas, A., Codony, X., Buschmann, H. (2008). Selective 5-HT_6 receptor ligands: progress in the development of a novel pharmacological approach to the treatment of obesity and related metabolic disorders. *Pharmacology and Therapeutics* **117**, 207-231.

Healy, D. J., Meador-Woodruff, J. H. (1999). Ionotropic glutamate receptor modulation of 5-HT_6 and 5-HT_7 mRNA expression in rat brain. *Neuropsychopharmacology* **21**, 341-351.

Hernandez, C. M., Gearhart, D. A., Parikh, V., Hohnadel, E. J., Davis, L. W., Middlemore, M. L., Warsi, S. P., Waller, J. L., Terry, A. V. (2006). Comparison of galantamine and donepezil for effects on nerve growth factor, cholinergic markers, and memory performance in aged rats. *Journal of Pharmacology and Experimental Therapeutics* **316**, 679-694.

Hirst, W. D., Abrahamsen, B., Blaney, F. E., Calver, A. R., Aloj, L., Price, G. W., Medhurst, A. D. (2003). Differences in the central nervous system distribution and pharmacology of the mouse 5-hydroxytryptamine-6 receptor compared with rat and human receptors investigated by radioligand binding, site-directed mutagenesis, and molecular modeling. *Molecular Pharmacology* **64**, 1295-1308.

Hirst, W. D., Minton, J. A. L., Bromidge, S. M., Moss, S. F., Latter, A. J., Riley, G., Routledge, C., Middlemiss, D. N., Price, G. W. (2000). Characterization of [I-125]-SB-258585 binding to human recombinant and native 5-HT_6 receptors in rat, pig and human brain tissue. *British Journal of Pharmacology* **130**, 1597-1605.

Hirst, W. D., Stean, T. O., Rogers, D. C., Sunter, D., Pugh, P., Moss, S. F., Bromidge, S. M., Riley, G., Smith, D. R., Bartlett, S., Heidbreder, C. A., Atkins, A. R., Lacroix, L. P., Dawson, L. A., Foley, A. G., Regan, C. M., Upton, N. (2006). SB-399885 is a potent, selective 5-HT_6 receptor antagonist with cognitive enhancing properties in aged rat water maze and novel object recognition models. *European Journal of Pharmacology* **553**, 109-119.

Hoyer, D., Clarke, D. E., Fozard, J. R., Hartig, P. R., Martin, G. R., Mylecharane, E. J., Saxena, P. R., Humphrey, P. P. A. (1994). International Union of Pharmacology Classification of Receptors for 5-Hydroxytryptamine (Serotonin). *Pharmacological Reviews* **46**, 157-203.

Huerta-Rivas, A., Perez-Garcia, G., Gonzalez-Espinosa, C., Meneses, A. (2010). Time-course of 5-HT_6 receptor mRNA expression during memory consolidation and amnesia. *Neurobiology of Learning and Memory* **93**, 99-110.

Humphrey, P. P. A., Hartig, P., Hoyer, D. (1993). A proposed new nomenclature for 5-HT receptors. *Trends in Pharmacological Sciences* **14**, 233-236.

Ishiyama, T., Tokuda, K., Ishibashi, T., Ito, A., Toma, S., Ohno, Y. (2007. Lurasidone (SM-13496), a novel atypical antipsychotic drug, reverses MK-801-induced impairment of learning and memory in the rat passive-avoidance test. *European Journal of Pharmacology* **572**, 160-170.

Izquierdo, I., Medina, J. H. (1997). Memory formation: The sequence of biochemical events in the hippocampus and its connection to activity in other brain structures. *Neurobiology of Learning and Memory* **68**, 285-316.

Izquierdo, I., Quillfeldt, J. A., Zanatta, M. S., Quevedo, J., Schaeffer, E., Schmitz, P. K., Medina, J. H. (1997). Sequential role of hippocampus and amygdala, entorhinal cortex and parietal cortex in formation and retrieval of memory for inhibitory avoidance in rats. *European Journal of Neuroscience* **9**, 786-793.

Jafari-Sabet, M. (2006a). NMDA receptor antagonists antagonize the facilitatory effects of post-training intra-basolateral arnygdala NMDA and physostigmine on passive avoidance learning. *European Journal of Pharmacology* **529**, 122-128.

Jafari-Sabet, M. (2006b). NMDA receptor blockers prevents the facilitatory effects of post-training intra-dorsal hippocampal NMDA and physostigmine on memory retention of passive avoidance learning in rats. *Behavioural Brain Research* **169**, 120-127.

Ji, J., Maren, S. (2007). Hippocampal involvement in contextual modulation of fear extinction. *Hippocampus* **17**, 749-758.

Jones, K. W., Bauerle, L. M., Denoble, V. J. (1990). Differential effects of sigma receptor and phencyclidine receptor ligands on learning. *European Journal of Pharmacology* **179**, 97-102.

Kendall, I., Slotten, H. A., Codony, X., Burgueno, J., Pauwels, P. J., Vela, J. M., Fone, K. C. (2010). E-6801, a 5-HT_6 receptor agonist, improves recognition memory by combined modulation of cholinergic and glutamatergic neurotransmission in the rat. *Psychopharmacology (Berl)*.

Kim, J. J., Fanselow, M. S. (1992). Modality-specific retrograde amnesia of fear. *Science* **256**, 675-677.

Kim, J. J., Rison, R. A., Fanselow, M. S. (1993). Effects of amygdala, hippocampus, and periaqueductal gray lesions on short-term and long-term contextual fear. *Behavioral Neuroscience* **107**, 1093-1098.

Kim, M., McGaugh, J. L. (1992). Effects of intraamygdala injections of NMDA receptor antagonists on acquisition and retention of inhibitory avoidance. *Brain Research* **585**, 35-48.

King, M. V. (2006). The Role of the 5-HT_6 Receptor in Memory and Attention. *Thesis, University of Nottingham*.

King, M. V., Marsden, C. A., Fone, K. C. F. (2008). A role for the 5-HT_{1A}, 5-HT_4 and 5-HT_6 receptors in learning and memory. *Trends in Pharmacological Science* **29**, 482-492.

King, M. V., Sleight, A. J., Fone, K. C. F., Marsden, C. A. (2006). A 5-HT_6 receptor agonist prolongs memory in the novel object discrimination (NOD) task. *Journal of Psychopharmacology* **20S**, A66.

King, M. V., Sleight, A. J., Woolley, M. L., Topham, I. A., Marsden, C. A., Fone, K. C. F. (2004). 5-HT_6 receptor antagonists reverse delay-dependent deficits in novel object discrimination by enhancing consolidation - an effect sensitive to NMDA receptor antagonism. *Neuropharmacology* **47**, 195-204.

Knipper, M., da Penha Berzaghi, M., Blochl, A., Breer, H., Thoenen, H., Lindholm, D. (1994). Positive feedback between acetylcholine and the neurotrophins nerve growth factor and brain-derived neurotrophic factor in the rat hippocampus. *European Journal of Neuroscience* **6**, 668-671.

Kohen, R., Fashingbauer, L. A., Heidmann, D. E. A., Guthrie, C. R., Hamblin, M. W. (2001). Cloning of the mouse 5-HT_6 serotonin receptor and mutagenesis studies of the third cytoplasmic loop. *Molecular Brain Research* **90**, 110-117.

Kohen, R., Metcalf, M. A., Khan, N., Druck, T., Huebner, K., Lachowicz, J. E., Meltzer, H. Y., Sibley, D. R., Roth, B. L., Hamblin, M. W. (1996). Cloning, characterization, and chromosomal localization of a human 5-HT_6 serotonin receptor. *Journal of Neurochemistry* **66**, 47-56.

Korte, M., Carroll, P., Wolf, E., Brem, G., Thoenen, H., Bonhoeffer, T. (1995). Hippocampal long-term potentiation is impaired in mice lacking brain-derived neurotrophic factor. *Proceedings of the National Academy of Sciences U.S.A* **92**, 8856-8860.

Lacroix, L. P., Dawson, L. A., Hagan, J. J., Heidbreder, C. A. (2004). 5-HT_6 receptor antagonist SB-271046 enhances extracellular levels of monoamines in the rat medial prefrontal cortex. *Synapse* **51**, 158-164.

Lee, H. J., Berger, S. Y., Stiedl, O., Spiess, J., Kim, J. J. (2001). Post-training injections of catecholaminergic drugs do not modulate fear conditioning in rats and mice. *Neuroscience Letters* **303**, 123-126.

Lee, H. K., Barbarosie, M., Kameyama, K., Bear, M. F., Huganir, R. L. (2000). Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. *Nature* **405**, 955-959.

Lefkowitz, R. J., Caron, M. G. (1988). Adrenergic receptors-models for the study of receptor coupled to guanine-nucleotide regulatory proteins. *Journal of Biological Chemistry* **263**, 4993-4996.

Lewis, D. A., Moghaddam, B. (2006). Cognitive dysfunction in schizophrenia: convergence of gamma-aminobutyric acid and glutamate alterations. *Archives of Neurology* **63**, 1372-1376.

Li, H. B., Matsumoto, K., Tohda, M., Yamamoto, M., Watanabe, H. (1997). NMDA antagonists potentiate scopolamine-induced amnesic effect. *Behavioural Brain Research* **83**, 225-228.

Li, X. M., Perry, K. W., Wong, D. T., Bymaster, F. P. (1998). Olanzapine increases in vivo dopamine and norepinephrine release in rat prefrontal cortex, nucleus accumbens and striatum. *Psychopharmacology* **136**, 153-161.

Liang, K. C., Juler, R. G., McGaugh, J. L. (1986). Modulating effects of posttraining epinephrine on memory: involvement of the amygdala noradrenergic system. *Brain Research* **368**, 125-133.

Lieben, C. K., Blokland, A., Sik, A., Sung, E., van Nieuwenhuizen, P., Schreiber, R. (2005). The selective 5-HT_6 receptor antagonist Ro4368554 restores memory performance in cholinergic and serotonergic models of memory deficiency in the rat. *Neuropsychopharmacology* **30**, 2169-2179.

Lindner, M. D., Hodges, D. B., Hogan, J. B., Orie, A. F., Corsa, J. A., Barten, D. M., Polson, C., Robertson, B. J., Guss, V. L., Gillman, K. W., Starrett, J. E., Gribkoff, V. K. (2003). An assessment of the effects of serotonin 6 (5-HT_6) receptor antagonists in rodent models of learning. *Journal of Pharmacology and Experimental Therapeutics* **307**, 682-691.

Lindner, M. D., Hogan, J. B., Hodges, D. B., Jr., Orie, A. F., Chen, P., Corsa, J. A., Leet, J. E., Gillman, K. W., Rose, G. M., Jones, K. M., Gribkoff, V. K. (2006). Donepezil primarily attenuates scopolamine-induced deficits in psychomotor function, with moderate effects on simple conditioning and attention, and small effects on working memory and spatial mapping. *Psychopharmacology (Berl)* **188**, 629-640.

Liu, I. Y., Lyons, W. E., Mamounas, L. A., Thompson, R. F. (2004). Brain-derived neurotrophic factor plays a critical role in contextual fear conditioning. *Journal of Neuroscience* **24**, 7958-7963.

Loiseau, F., Dekeyne, A., Millan, M. J. (2008). Pro-cognitive effects of 5-HT_6 receptor antagonists in the social recognition procedure in rats: implication of the frontal cortex. *Psychopharmacology* **196**, 93-104.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951). Protein measurement with the foline phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.

Lu, Y., Christian, K., Lu, B. (2008). BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? *Neurobiology of Learning and Memory* **89**, 312-323.

Lynch, M. A. (2004). Long-term potentiation and memory. *Physiological Reviews* **84**, 87-136.

Malenka, R. C., Bear, M. F. (2004). LTP and LTD: an embarrassment of riches. *Neuron* **44**, 5-21.

Marcos, B., Aisa, B., Ramirez, M. J. (2008a). Functional interaction between 5-HT_6 receptors and hypothalamic-pituitary-adrenal axis: cognitive implications. *Neuropharmacology* **54**, 708-714.

Marcos, B., Chuang, T. T., Gil-Bea, F. J., Ramirez, M. J. (2008b). Effects of 5-HT_6 receptor antagonism and cholinesterase inhibition in models of cognitive impairment in the rat. *British Journal of Pharmacology* **155**, 434-440.

Marcos, B., Gil-Bea, F. J., Hirst, W. D., Garcia-Alloza, M., Ramirez, M. J. (2006). Lack of localization of 5-HT_6 receptors on cholinergic neurons: implication of multiple neurotransmitter systems in 5-HT_6 receptor-mediated acetylcholine release. *European Journal of Neuroscience* **24**, 1299-1306.

Maren, S. (2001). Neurobiology of Pavlovian fear conditioning. *Annual Review of Neuroscience* **24**, 897-931.

Maren, S., Aharonov, G., Fanselow, M. S. (1997). Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. *Behavioural Brain Research* **88**, 261-274.

Maren, S., Aharonov, G., Stote, D. L., Fanselow, M. S. (1996). N-methyl-D-aspartate receptors in the basolateral amygdala are required for both acquisition and expression of conditional fear in rats. *Behavioral Neuroscience* **110**, 1365-1374.

Maren, S., Fanselow, M. S. (1995). Synaptic plasticity in the basolateral amygdala induced by hippocampal formation stimulation in vivo. *Journal of Neuroscience* **15**, 7548-7564.

Marshall, E. F., Stirling, G. S., Tait, A. C., Todrick, A. (1960). Effect of iproniazid and imipramine on the blood platelet 5-hydroxytryptamine level in man. *British Journal of Pharmacology and Chemotherapy* **15**, 35-41.

Martin, S. J., Grimwood, P. D., Morris, R. G. (2000). Synaptic plasticity and memory: an evaluation of the hypothesis. *Annual Review of Neuroscience* **23**, 649-711.

Mattsson, C., Sonesson, C., Sandahl, A., Greiner, H. E., Gassen, M., Plaschke, J., Leibrock, J., Bottcher, H. (2005). 2-Alkyl-3-(1,2,3,6-tetrahydropyridin-4-yl)-1H-indoles as novel 5-HT_6 receptor agonists. *Bioorganic and Medicinal Chemistry Letters* **15**, 4230-4234.

Max, S. L., Monsma, F. J., Sibley, D. R. (1995). Agonist-induced desensitisation of the 5-HT_6 serotonin receptor-coupled adenylyl cyclase in stably transfected HEK-293 cells. *Journal of Serotonin Research*. **2**, 101-116.

McGaugh, J. L. (2002). Memory consolidation and the amygdala: a systems perspective. *Trends in Neuroscience* **25**, 456.

Meneses, A. (2001). Effects of the 5-HT_6 receptor antagonist Ro 04-6790 on learning consolidation. *Behavioural Brain Research* **118**, 107-110.

Meneses, A., Manuel-Apolinar, L., Castillo, C., Castillo, E. (2007). Memory consolidation and amnesia modify 5-HT_6 receptors expression in rat brain: an autoradiographic study. *Behavioral Brain Research* **178**, 53-61.

Meneses, A., Perez-Garcia, G., Liy-Salmeron, G., Flores-Galvez, D., Castillo, C., Castillo, E. (2008). The effects of the 5-HT_6 receptor agonist EMD and the 5-HT_7 receptor agonist AS19 on memory formation. *Behavioral Brain Research* **195**, 112-119.

Mikami, A., Masuoka, T., Yasuda, M., Yamamoto, Y., Kamei, C. (2007). Participation of cholinergic system in memory deficits induced by blockade of hippocampal mGlu(1) receptors. *European Journal of Pharmacology* **575**, 82-86.

Milner, B., Squire, L. R., Kandel, E. R. (1998). Cognitive neuroscience and the study of memory. *Neuron* **20**, 445-468.

Mishima, K., Iwasaki, K., Tsukikawa, H., Matsumoto, Y., Egashira, N., Abe, K., Egawa, T., Fujiwara, M. (2000). The scopolamine-induced impairment of spatial cognition parallels the acetylcholine release in the ventral hippocampus in rats. *The Japanese Journal of Pharmacology* **84**, 163-173.

Mitchell, E. S., Hoplight, B. J., Lear, S. P., Neumaier, J. F. (2006). BGC20-761, a novel tryptamine analog, enhances memory consolidation and reverses scopolamine-induced memory deficit in social and visuospatial memory tasks through a 5-HT_6 receptor-mediated mechanism. *Neuropharmacology* **50**, 412-420.

Mitchell, E. S., Neumaier, J. F. (2005). 5-HT_6 receptors: a novel target for cognitive enhancement. *Pharmacology & Therapeutics* **108**, 320-333.

Mitchell, E. S., Neumaier, J. F. (2008). 5-HT_6 receptor antagonist reversal of emotional learning and prepulse inhibition deficits induced by apomorphine or scopolamine. *Pharmacology Biochemistry and Behavior* **88**, 291-298.

Mizuno, K., Giese, K. P. (2005). Hippocampus-dependent memory formation: do memory type-specific mechanisms exist? *Journal of Pharmacological Sciences* **98**, 191-197.

Mizuno, M., Yamada, K., Olariu, A., Nawa, H., Nabeshima, T. (2000). Involvement of brain-derived neurotrophic factor in spatial memory formation and maintenance in a radial arm maze test in rats. *Journal of Neuroscience* **20**, 7116-7121.

Monsma, F. J., Jr., Shen, Y., Ward, R. P., Hamblin, M. W., Sibley, D. R. (1993). Cloning and expression of a novel serotonin receptor with high affinity for tricyclic psychotropic drugs. *Molecular Pharmacology* **43**, 320-327.

Monti, B., Berteotti, C., Contestabile, A. (2005). Dysregulation of memory-related proteins in the hippocampus of aged rats and their relation with cognitive impairment. *Hippocampus* **15**, 1041-1049.

Morris, R. G. M., Anderson, E., Lynch, G. S., Baudry, M. (1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. *Nature* **319**, 774-776.

Murchison, C. F., Zhang, X. Y., Zhang, W. P., Ouyang, M., Lee, A., Thomas, S. A. (2004). A distinct role for norepinephrine in memory retrieval. *Cell* **117**, 131-143.

Nail-Boucherie, K., Dourmap, N., Jaffard, R., Costentin, J. (2000). Contextual fear conditioning is associated with an increase of acetylcholine release in the hippocampus of rat. *Brain Research Cognitive Brain Research* **9**, 193-197.

Nadel, L., Moscovitch, M. (1997). Memory consolidation, retrograde amnesia and the hippocampal complex. *Current Opinion in Neurobiology* **7**, 217-227.

Nestor, P. J., Fryer, T. D., Hodges, J. R. (2006). Declarative memory impairments in Alzheimer's disease and semantic dementia. *Neuroimage* **30**, 1010-1020.

Nilsson, M., Hansson, S., Carlsson, A., Carlsson, M. L. (2007). Differential effects of the N-methyl-D-aspartate receptor antagonist MK-801 on different stages of object recognition memory in mice. *Neuroscience* **149**, 123-130.

Ohmori, O., Shinkai, T., Hori, H., Nakamura, J. (2001). Novel polymorphism in the 5 '-upstream region of the human 5-HT_6 receptor gene and schizophrenia. *Neuroscience Letters* **310**, 17-20.

Olverman, H. J., Jones, A. W., Watkins, J. C. (1984). L-Glutamate has higher affinity than other amino acids for [H-3] D-AP5 binding sites in rat brain membranes. *Nature* **307**, 460-462.

Orsetti, M., Casamenti, F., Pepeu, G. (1996). Enhanced acetylcholine release in the hippocampus and cortex during acquisition of an operant behavior. *Brain Research* **724**, 89-96.

Otano, A., Frechilla, D., Cobreros, A., Cruz-Orive, L. M., Insausti, A., Insausti, R., Hamon, M., Del Rio, J. (1999). Anxiogenic-like effects and reduced stereological counting of immunolabelled 5-hydroxytryptamine(6) receptors in rat nucleus accumbens by antisense oligonucleotides. *Neuroscience* **92**, 1001-1009.

Pastalkova, E., Serrano, P., Pinkhasova, D., Wallace, E., Fenton, A. A., Sacktor, T. C. (2006). Storage of spatial information by the maintenance mechanism of LTP. *Science* **313**, 1141-1144.

Pavlov, I. P. (1927). Conditioned Reflexex. An Investigation of the Physiological Activity of the Cerebral Cortex. Oxford University Press.

Pedigo, N. W., Yamamura, H. I., Nelson, D. L. (1981). Discrimination of multiple [H-3]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. *Journal of Neurochemistry* **36**, 220-226.

Pepeu, G., Giovannini, M. G. (2009). Changes in acetylcholine extracellular levels during cognitive processes. *Learning and Memory* **11**, 21-27.

Perez-Garcia, G., Meneses, A. (2005). Oral administration of the 5-HT_6 receptor antagonists SB-357134 and SB-399885 improves memory formation in an autoshaping learning task. *Pharmacology Biochemistry and Behavior* **81**, 673-682.

Peroutka, S. J., Snyder, S. H. (1979). Multiple serotonin receptors-differential binding of [5-hydroxytryptamine-H-3, [lysergic-H-3 acid diethylamide and [H-3] spiroperidol. *Molecular Pharmacology* **16**, 687-699.

Perry, E. K., Johnson, M., Kerwin, J. M., Piggott, M. A., Court, J. A., Shaw, P. J., Ince, P. G., Brown, A., Perry, R. H. (1992). Convergent cholinergic activities in aging and Alzheimers Disease. *Neurobiology of Aging* **13**, 393-400.

Perry, E. K., Tomlinson, B. E., Blessed, G., Bergmann, K., Gibson, P. H., Perry, R. H. (1978). Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *British Medical Journal* **2**, 1457-1459.

Phillips, R. G., Ledoux, J. E. (1992). Differential Contribution of Amygdala and Hippocampus to Cued and Contextual Fear Conditioning. *Behavioral Neuroscience* **106**, 274-285.

Phillips, R. G., Ledoux, J. E. (1995). Lesions of the fornix but not the entorhinal or perirhinal cortex interfere with contextual fear conditioning. *Journal of Neuroscience* **15**, 5308-5315.

Pitsikas, N., Zisopouiou, S., Pappas, I., Sakellaridis, N. (2008). The selective 5-HT_6 receptor antagonist Ro 04-6790 attenuates psychotomimetic effects of the NMDA receptor antagonist MK-801. *Behavioural Brain Research* **188**, 304-309.

Pizzorusso, T. (2009). Erasing Fear Memories. *Science* **325**, 1214-1215.

Pletscher, A., Shore, P. A., Brodie, B. B. (1955). Serotonin release as a possible mechanism of reserpine action. *Science* **122**, 374-375.

Plaznik, A., Danysz, W., Kostowski, W. (1985). Some behavioral effects of microinjections of noradrenaline and serotonin into the amygdaloid body of the rat brain. *Physiology & Behavior* **34**, 481-487.

Pouzet, B., Didriksen, M., Arnt, J. (2002). Effects of the 5-HT_6 receptor antagonist, SB-271046, in animal models for schizophrenia. *Pharmacology Biochemistry and Behavior* **71**, 635-643.

Pullagurla, M. R., Westkaemper, R. B., Glennon, R. A. (2004). Possible differences in modes of agonist and antagonist binding at human 5-HT_6 receptors. *Bioorganic & Medicinal Chemistry Letters* **14**, 4569-4573.

Rapport, M. M., Green, A. A., Page, I. H. (1948a). Crystalline Serotonin. *Science* **108**, 329-330.

Rapport, M. M., Green, A. A., Page, I. H. (1948b). Partial purification of the vasoconstrictor in beef serum. *Journal of Biological Chemistry* **174**, 735-741.

Rapport, M. M., Green, A. A., Page, I. H. (1948c). Serum Vasoconstrictor (Serotonin). 4. Isolation and Characterization. *Journal of Biological Chemistry* **176**, 1243-1251.

Rapport, M. M. (1949). Serum vasoconstrictor (serotonin) the presence of creatinine in the complex; a proposed structure of the vasoconstrictor principle. *Journal of Biological Chemistry* **180**, 961-969.

Rattiner, L. M., Davis, M., Ressler, K. J. (2004). Differential regulation of brain-derived neurotrophic factor transcripts during the consolidation of fear learning. *Learning and Memory* **11**, 727-731.

Riemer, C., Borroni, E., Levet-Trafit, B., Martin, J. R., Poli, S., Porter, R. H., Bos, M. (2003). Influence of the 5-HT_6 receptor on acetylcholine release in the cortex: pharmacological characterization of 4-(2-bromo-6-pyrrolidin-1-ylpyridine-4-sulfonyl)phenylamine, a potent and selective 5-HT_6 receptor antagonist. *Journal of Medicinal Chemistry* **46**, 1273-1276.

Rogers, D. C., Hagan, J. J. (2001). 5-HT_6 receptor antagonists enhance retention of a water maze task in the rat. *Psychopharmacology (Berl)* **158**, 114-119.

Romero, G., Sanchez, E., Pujol, M., Perez, P., Codony, X., Holenz, J., Buschmann, H., Pauwels, P. J. (2006). Efficacy of selective 5-HT_6 receptor ligands determined by monitoring 5-HT_6 receptor-mediated cAMP signaling pathways. *British Journal of Pharmacology* **148**, 1133-1143.

Roth, B. L., Craigo, S. C., Choudhary, M. S., Uluer, A., Monsma, F. J., Shen, Y., Meltzer, H. Y., Sibley, D. R. (1994). Binding of typical and atypical antipsychotic agents to 5-hydroxytryptamine-6 and 5-hydroxytryptamine-7 receptors. *Journal of Pharmacology and Experimental Therapeutics* **268**, 1403-1410.

Roth, B. L., Hanizavareh, S. M., Blum, A. E. (2004). Serotonin receptors represent highly favorable molecular targets for cognitive enhancement in schizophrenia and other disorders. *Psychopharmacology* **174**, 17-24.

Routledge, C., Bromidge, S. M., Moss, S. F., Price, G. W., Hirst, W., Newman, H., Riley, G., Gager, T., Stean, T., Upton, N., Clarke, S. E., Brown, A. M., Middlemiss, D. N. (2000). Characterization of SB-271046: A potent, selective and orally active 5-HT_6 receptor antagonist. *British Journal of Pharmacology* **130**, 1606-1612.

Ruat, M., Traiffort, E., Arrang, J. M., Tardivel-Lacombe, J., Diaz, J., Leurs, R., Schwartz, J. C. (1993). A novel rat serotonin (5-HT_6) receptor: molecular cloning, localization and stimulation of cAMP accumulation. *Biochemical and Biophysical Research Communications* **193**, 268-276.

Russell, M. G. N., Baker, R. J., Barden, L., Beer, M. S., Bristow, L., Broughton, H. B., Knowles, M., McAllister, G., Patel, S., Castro, J. L. (2001). N-Arylsulfonulindole derivatives as serotonin 5-HT_6 receptor ligands. *Journal of Medicinal Chemistry* **44**, 3881-3895.

Russell, M. G. N., Dias, R. (2002). Memories are made of this (perhaps): A review of serotonin 5-HT_6 receptor ligands and their biological functions. *Current Topics in Medicinal Chemistry* **2**, 643-654.

Schechter, L. E., Lin, Q., Smith, D. L., Zhang, G. M., Shan, Q., Platt, B., Brandt, M. R., Dawson, L. A., Cole, D., Bernotas, R., Robichaud, A., Rosenzweig-Lipson, S., Beyer, C. E. (2008). Neuropharmacological profile of novel and selective 5-HT_6 receptor agonists: WAY-181187 and WAY-208466. *Neuropsychopharmacology* **33**, 1323-1335.

Schechter, L. E., Ring, R. H., Beyer, C. E., Hughes, Z. A., Khawaja, X., Malberg, J. E., Rosenzweig-Lipson, S. (2005). Innovative approaches for the development of antidepressant drugs: current and future strategies. *NeuroRx* **2**, 590-611.

Schreiber, R., Vivian, J., Hedley, L., Szczepanski, K., Secchi, R. L., Zuzow, M., van Laarhoven, S., Moreau, J. L., Martin, J. R., Sik, A., Blokland, A. (2007). Effects of the novel 5-HT_6 receptor antagonist RO4368554 in rat models for cognition and sensorimotor gating. *European Neuropsychopharmacology* **17**, 277-288.

Scoville, W. B., Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *Journal of Neurology, Neurosurgery and Psychiatry* **20**, 11-21

Shaw, E., Woolley, D. W. (1953). Yohimbine and ergot alkaloids as naturally occurring antimetabolites of serotonin. *Journal of Biological Chemistry* **203**, 979-989.

Shirazi-Southall, S., Rodriguez, D. E., Nomikos, G. G. (2002). Effects of typical and atypical antipsychotics and receptor selective compounds on acetylcholine efflux in the hippocampus of the rat. *Neuropsychopharmacology* **26**, 583-594.

Sleight, A. J., Boess, F. G., Bos, M., Levet-Trafit, B., Riemer, C., Bourson, A. (1998). Characterization of Ro 04-6790 and Ro 63-0563: potent and selective antagonists at human and rat 5-HT_6 receptors. *British Journal of Pharmacology* **124**, 556-562.

Sleight, A. J., Monsma, F. J., Borroni, E., Austin, R. H., Bourson, A. (1996). Effects of altered 5-ht(6) expression in the rat: Functional studies using antisense oligonucleotides. *Behavioural Brain Research* **73**, 245-248.

Squire, L. R. (2004). Memory systems of the brain: a brief history and current perspective. *Neurobiology of Learning and Memory* **82**, 171-177.

Stean, T. O., Hirst, W. D., Thomas, D. R., Price, G. W., Rogers, D., Riley, G., Bromidge, S. M., Serafinowska, H. T., Smith, D. R., Bartlett, S., Deeks, N., Duxon, M., Upton, N. (2002). Pharmacological profile of SB-357134: A potent, selective, brain penetrant, and orally active 5-HT_6 receptor antagonist. *Pharmacology Biochemistry and Behavior* **71**, 645-654.

Steinbusch, H. W. M. (1981). Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience* **6**, 557-618.

Sunderland, T., Tariot, P. N., Weingartner, H., Murphy, D. L., Newhouse, P. A., Mueller, E. A., Cohen, R. M. (1986). Pharmacological modeling of Alzheimer's disease. *Progress in Neuro-Psychopharmacology & Biological Psychiatry* **10**, 599-610.

Suzuki, W. A., Eichenbaum, H. (2000). The neurophysiology of memory. *Annals of the New York Academy of Science* **911**, 175-191.

Svenningsson, P., Tzavara, E. T., Qi, H., Carruthers, R., Witkin, J. M., Nomikos, G. G., Greengard, P. (2007). Biochemical and behavioral evidence for antidepressant-like effects of 5-HT_6 receptor stimulation. *Journal of Neuroscience* **27**, 4201-4209.

Tamir, H., Gershon, H. D. (1979). Storage of serotonin and serotonin-binding protein in synaptic vesicles. *Journal of Neurochemistry* **32**, 593-598.

Terry, A. V., Jr., Buccafusco, J. J., Wilson, C. (2008). Cognitive dysfunction in neuropsychiatric disorders: selected serotonin receptor subtypes as therapeutic targets. *Behavioral Brain Research* **195**, 30-38.

Thompson, R. F. (1986). The Neurobiology of Learning and Memory. *Science* **233**, 941-947.

Tronson, N. C., Schrick, C., Fischer, A., Sananbenesi, F., Pages, G., Pouyssegur, J., Radulovic, J. (2008). Regulatory mechanisms of fear extinction and depression-like behavior. *Neuropsychopharmacology* **33**, 1570-1583.

Tsai, Y. C., Dukat, M., Slassi, A., MacLean, N., Demchyshyn, L., Savage, J. E., Roth, B. L., Hufesein, S., Lee, M., Glennon, R. A. (2000). N-1-(benzenesulfonyl)tryptamines as novel 5-HT_6 antagonists. *Bioorganic & Medicinal Chemistry Letters* **10**, 2295-2299.

Tsai, S., Liu, H., Liu, T., Wang, Y.,Hong, C. (1999). Association analyses of the 5-HT_6 receptor polymorphism C267T in Alzheimer's disease. *Neuroscience Letters* **276**, 138-139.

Twarog, B. M., Page, I. H., Bailey, H. (1953). Serotonin content of some mammalian tissues and urine and a method for its determination. *American Journal of Physiology* **175**, 157-161.

Upton, N., Chuang, T. T., Hunter, A. J., Virley, D. J. (2008). 5-HT_6 receptor antagonists as novel cognitive enhancing agents for Alzheimer's disease. *Neurotherapeutics* **5**, 458-469.

Upton, N., Stean, T., Middlemiss, D., Blackburn, T., Kennett, G. (1998). Studies on the role of 5-HT_{2C} and 5-HT_{2B} receptors in regulating generalised seizure threshold in rodents. *European Journal of Pharmacology* **359**, 33-40.

Vogt, I. R., Shimron-Abarbanell, D., Neidt, H., Erdmann, J., Cichon, S., Schulze, T. G., Muller, D. J., Maier, W., Albus, M., Borrmann-Hassenbach, M., Knapp, M., Rietschel, M., Propping, P., Nothen, M. M. (2000). Investigation of the human serotonin 6 (5-HT_6) receptor gene in bipolar affective disorder and schizophrenia. *American Journal of Medical Genetics* **96**, 217-221.

Wang, H., Hu, Y., Tsien, J. Z. (2006). Molecular and systems mechanisms of memory consolidation and storage. *Progress in Neurobiology* **79**, 123-135.

Ward, R. P., Hamblin, M. W., Lachowicz, J. E., Hoffman, B. J., Sibley, D. R., Dorsa, D. M. (1995). Localization of serotonin subtype 6 receptor messenger RNA in the rat brain by in situ hybridization histochemistry. *Neuroscience* **64**, 1105-1111.

Weissbach, H., Redfield, B. G., Lovenberg, W., Udenfriend, S. (1961). In vivo metabolism of serotonin and tryptamine-effect of monoamine oxidase inhibition. *Journal of Pharmacology and Experimental Therapeutics* **131**, 26-&.

Wesolowska, A. (2008). The anxiolytic-like effect of the selective 5-HT_6 receptor antagonist SB-399885: the impact of benzodiazepine receptors. *European Journal of Pharmacology* **580**, 355-360.

Wesolowska, A., Nikiforuk, A. (2007). Effects of the brain-penetrant and selective 5-HT_6 receptor antagonist SB-399885 in animal models of anxiety and depression. *Neuropharmacology* **52**, 1274-1283.

Wesolowska, A., Nikiforuk, A., Stachowicz, K. (2007). Anxiolytic-like and antidepressant-like effects produced by the selective 5-HT_6 receptor antagonist SB-258585 after intrahippocampal administration to rats. *Behavioural Pharmacology* **18**, 439-446.

Whittaker-Azmitia, P. M. (1999). The discovery of serotonin and its role in neuroscience. *Neuropsychopharmacology* **21**, S2-S8.

Whitlock, J. R., Heynen, A. J., Shuler, M. G., Bear, M. F. (2006). Learning induces long-term potentiation in the hippocampus. *Science* **313**, 1093-1097.

Wilensky, A. E., Schafe, G. E., LeDoux, J. E. (1999). Functional inactivation of the amygdala before but not after auditory fear conditioning prevents memory formation. *Journal of Neuroscience* **19**, RC48.

Wiltgen, B. J., Brown, R. A. M., Talton, L. E., Silva, A. J. (2004). New circuits for old memories: The role of the neocortex in consolidation. *Neuron* **44**, 101-108.

Woolley, D. W., Shaw, E. (1954). A biochemical and pharmacological suggestion about certain mental disorders. *Proceedings of the National Academy of Sciences of the United States of America* **40**, 228-231.

Woolley, M.L. (2002). The Functional Role of the 5-HT_6 Receptor in the Rat CNS. *Thesis, University of Nottingham*.

Woolley, M. L., Bentley, J. C., Sleight, A. J., Marsden, C. A., Fone, K. C. F. (2001). A role for 5-ht(6) receptors in retention of spatial learning in the Morris water maze. *Neuropharmacology* **41**, 210-219.

Woolley, M. L., Marsden, C. A., Fone, K. C. F. (2004). 5-ht_6 receptors. *Current Drug Targets - CNS and Neurological Disorders* **3**, 59-79.

Woolley, M. L., Marsden, C. A., Sleight, A. J., Fone, K. C. (2003). Reversal of a cholinergic-induced deficit in a rodent model of recognition memory by the selective 5-HT_6 receptor antagonist, Ro 04-6790. *Psychopharmacology (Berl)* **170**, 358-367.

Yau, J. L. W., Noble, J., Widdowson, J., Seckl, J. R. (1997). Impact of adrenalectomy on 5-HT_6 and 5-HT_7 receptor gene expression in the rat hippocampus. *Molecular Brain Research* **45**, 182-186.

Yoshioka, M., Matsumoto, M., Togashi, H., Mori, K., Saito, H. (1998). Central distribution and function of 5-HT_6 receptor subtype in the rat brain. *Life Science* **62**, 1473-1477.

Young, S. L., Bohenek, D. L., Fanselow, M. S. (1995). Scopolamine impairs acquisition and facilitates consolidation of fear conditioning: differential effects for tone vs context conditioning. *Neurobiology of Learning and Memory* **63**, 174-180.

Yuede, C. M., Dong, H. X., Csernansky, J. G. (2007). Anti-dementia drugs and hippocampal-dependent memory in rodents. *Behavioural Pharmacology* **18**, 347-363.

Yun, H. M., Kim, S., Kim, H. J., Kostenis, E., Kim, J. I., Seong, J. Y., Baik, J. H., Rhim, H. (2007). The novel cellular mechanism of human 5-HT_6 receptor through an interaction with Fyn. *Journal of Biological Chemistry* **282**, 5496-5505.

Zhukovskaya, N. L., Neumaier, J. F. (2000). Clozapine downregulates 5-hydroxytryptamine(6) (5-HT_6) and upregulates 5-HT_7 receptors in HeLa cells. *Neuroscience Letters* **288**, 236-240.

Zolamorgan, S. M., Squire, L. R. (1990). The primate hippocampal-formation-evidence for a time-limited role in memory storage. *Science* **250**, 288-290.